A CARDIO-DEPRESSOR SUBSTANCE (ACETYLCHOLINE?) RELEASED BY DORSAL NERVE ROOT STIMULATION

by

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It has been suggested by Dale (1929) that vaso-dilatation in response to stimulation of vaso-dilator nerves is caused by the local release of acetylcholine from the nerve endings. Subsequently Dale and Gaddum (1930) demonstrated the remarkable parallelism between the effects which are produced in denervated voluntary muscle by acetylcholine and by stimulation of its vaso-dilator nerves. Finally Feldberg (1933) has found that stimulation of the vaso-dilator nerve (lingual) to the dog's tongue confers upon the blood passing through it the property of (i.) lowering the dog's blood-pressure, (ii.) contracting sensitized leech muscle, provided always that the dog has undergone a previous injection of eserine.

The present paper describes experiments on the frog's hind limb in which the release of such a substance through dorsal nerve root stimulation has been sought; Doi (1920), and Krogh, Harrop, and Rehberg (1922) having already shown that the stimulation of these nerves causes vaso-dilatation in the frog. As an acetylcholine indicator the frog's isolated heart has been used, on which it acts as a depressant. No claim can, of course, be made for the specificity of the test, though the action of atropine and of eserine point to acetylcholine.

METHOD.

The experiments have all been made on frogs *(Hyla aurea)*.

The isolated heart is perfused continuously with the perfusate emerging steadily from the hind limbs of another frog (donor frog), and the effect is examined of dorsal nerve root stimulation of the latter on the contractions of the former.

(a) *Heart Preparation.*—The heart with a small portion of its vena cava is removed from a freshly-killed frog and fastened on to the floor of a small paraffine-lined dish, which is then filled with Ringer fluid. To obtain a steady beat it is imperative that the rate of filling of the sinus shall be constant. To secure this a larger vessel, which serves as a reservoir (R, Fig. 1) is filled to a height of about

2 cm. above that in the heart container. The perfusing fluid is transferred from the reservoir to the heart by an appropriately-bent capillary siphon, one end of

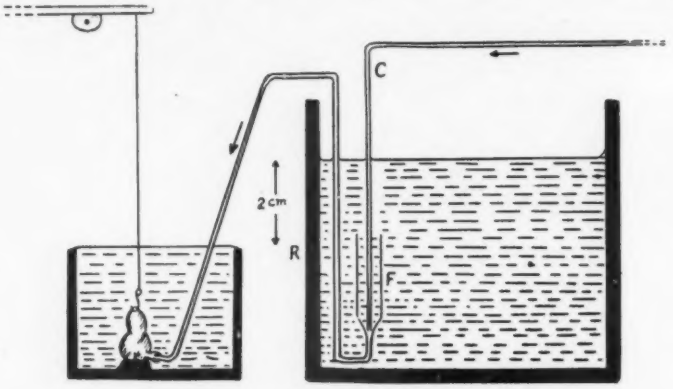

Figure 1. Description in text.

which is tied into the vena cava of the heart preparation, while the other end, expanded into a funnel (F), rests on the floor of the reservoir. The object of the funnel is to receive later a capillary cannula (C), carrying the perfusate from the donor, so preventing dilution by the large bulk of fluid in the reservoir. In these circumstances the heart preparation, after some preliminary fluctuation, contracts with great regularity for many hours. Its contractions are recorded on a slowly-rotating drum by an isotonic lever giving 20-fold magnification.

(b) *Donor Preparation.*—A frog is decapitated, and eviscerated, but not skinned. The aorta is preserved. The cauda equina is exposed by removing the centra of the vertebral column; the ventral roots are dissected away. The preparation is then perfused with Ringer fluid (phosphate buffered; pH 7·3), through a cannula inserted along the aorta. A head of about five or six inches of fluid in the cannula usually maintains an efficient flow. The renal portal vein having been ligated, the perfusate emerges entirely by the abdominal vein. A capillary cannula is inserted along this vein; the other end of this cannula (C, Fig. 3) is then fitted into the funnel of the siphoning tube through which the heart is perfused. A pair of fine, platinum-tipped electrodes connected with an induction coil is now fastened under the exposed dorsal nerve roots.

With the perfusion current running steadily through the donor into the receptor heart, the dorsal nerve roots of the former are given, periodically, prolonged stimulation, and the effect on the heart is observed.

RESULTS.

(1) *Response of the heart to dorsal root stimulation of the donor.*—When the donor is coupled with the heart preparation there usually occurs an almost im-

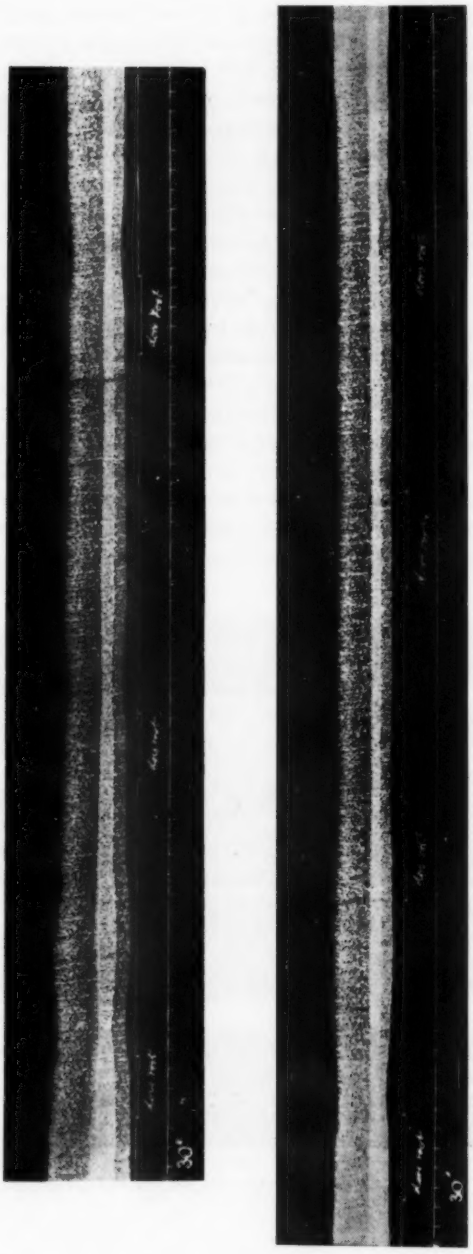

Figure 2. Seven successive responses of a heart to dorsal nerve root stimulation of donor preparation. Lower tracing is continuation of upper. Time in half-minutes. 15°C.

mediate increase in strength of beat; but this soon subsides and thereafter the heart gradually settles down to a steady and usually rather weakened beat. Dorsal root stimulation may then be started. The effect of such stimulation is illustrated by an experiment of which Figure 2 is a record. About a minute after commencement of stimulation the contractions begin to weaken. The depression is at its maximum after about one minute, the heart then gradually recovering again. The duration of the response is about three minutes. When dorsal root stimulation is repeated seven minutes later, the effect recurs though with noticeably less intensity. As Figure 2 shows it may be obtained as many as seven times in succession, though always with gradually diminishing intensity.

On the basis of many experiments the following general remarks can be made: (i.) in many preparations the effect fails to occur. No explanation can be offered for the frequent failure. Insensitiveness of the heart is not the sole explanation, for if several donors are successively coupled with one and the same heart some produce the effect, while with others it fails to occur. The similar uncertainty with which vagal effects can be transferred on to a second heart has already been stressed by Loewi (1924). (ii.) With some donors the effect is only very weak; (iii.) but if a particular donor produces a good initial effect on the heart, it can be relied upon to give a succession of effects on the same heart, though always with gradually decreasing intensity (Fig. 2).

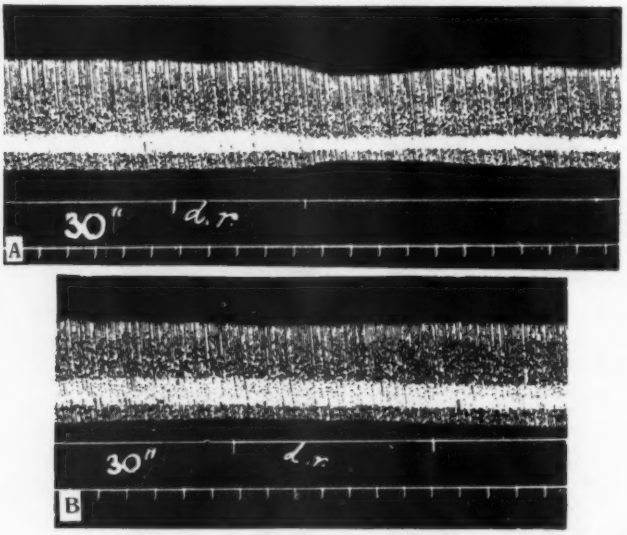

Figure 3. Action of atropine. A: normal response. B: the same, after atropine 1:100,000 (10 minutes). Time in half-minutes. 16°C.

It has occasionally been noticed that the recovery of a heart from a response may be incomplete, the heart remaining thereafter in a partially depressed state, e.g. Fig. 4A.

(2) *Action of Atropine.*—The action of atropine has been examined in two forms of experiment: (a) by passage through the donor into the heart; (b) by direct application to the heart, without passage through the donor.

In the former type of experiment, a preparation is selected in which the heart gives a good response to dorsal root stimulation (Fig. 3A). The fluid in the aortic cannula perfusing the donor is then replaced by atropine 1: 100,000. Ten minutes later dorsal root stimulation is without effect on the heart (Fig. 3B). The experiment in this form is made in the continued presence of atropine. In drawing an analogy between the paralysis of an acetylcholine response by means of atropine and the paralysis of a response to the substance released by dorsal nerve root stimulation, it may be objected that the atropine has prevented the liberation of the substance, and not abolished its action on the heart. For this purpose the experiment has been modified by perfusing atropine into the heart alone for a period of ten minutes, the donor being disconnected for the time. The donor is then coupled to the heart; five minutes later when the contractions have become steady dorsal root stimulation is completely without effect, although before atropine good effects were obtained.

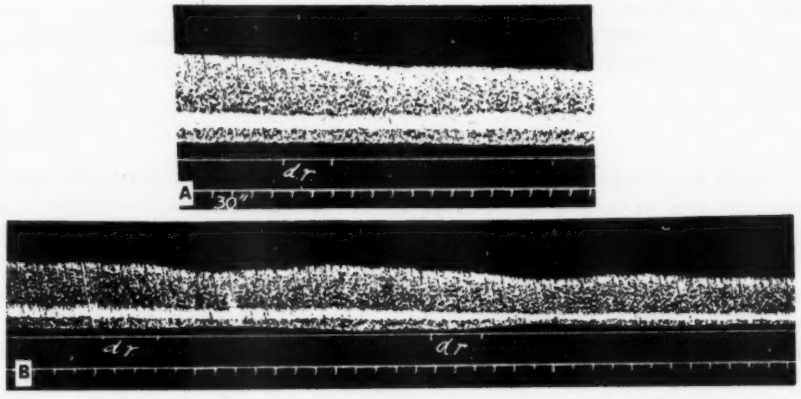

Figure 4. Action of eserine. A: normal response (very weak). B: the same after eserine 1:30,000 (10 minutes). Time in half-minutes. 18°C.

(3) *Action of Eserine.*—The action of eserine in augmenting the parasympathico-mimetic effects of acetylcholine seems to be due to inhibition of acetylcholine hydrolysis, owing to its injurious effect on tissue esterases (Führer, 1918; Loewi and Navratil, 1926; Matthes, 1930).

The present experiments show that eserine exerts such an augmenting action on the effect which is transferred on to the heart.

The experiment of which Figure 4 is a record will serve to illustrate this. The initial response of the heart to 1¼ minute dorsal root stimulation is exceedingly weak, lasting about 3 minutes (Fig. 4A). The perfusing fluid in the aorta cannula is now changed to Ringer containing eserine 1 : 30,000 (physostigmine salicylate, Burroughs Wellcome), which is perfused for the remainder of the experiment. Ten minutes after commencement of application of eserine the dorsal roots are stimulated again for 1¼ minutes; a powerful effect of 5 minutes' duration occurs, which may be repeated 7 minutes later (Fig. 4B).

Since with repeated stimulation the effects normally never increase, but on the contrary decrease, in intensity (Fig. 2) an augmenting effect of eserine seems clearly demonstrated.

(4) *Effect of Skinning the Limb.*—A number of experiments were performed in which the skin was removed during the course of the experiment, in preparations which had previously given a good response on the heart. In all cases dorsal root stimulation after skinning was without effect, even with eserine. It would seem then that the cardio-depressor substance is derived chiefly from the skin; but the possibility of its formation in the muscles in quantity too small to be detected by the heart cannot be excluded.

Doi (1920) found that vaso-dilatation due to dorsal root stimulation occurred mainly in the skin, the effect being only very feeble after its removal. Other evidence for dilatation of intramuscular blood vessels is summarized by Feldberg and Schilf (1930).

DISCUSSION.

From the foregoing it is evident that dorsal nerve root stimulation releases some substance in the skin of the frog, capable of depressing the beat of an isolated heart.

Depression of the heart can easily be produced by an increase in hydrogen ion concentration. But this could scarcely be the cause of the effect observed, for there is no reason to believe that dorsal nerve root stimulation can cause such a change in hydrogen ion concentration; nor would the effect of this be abolished by atropine or augmented by eserine. It seemed, nevertheless, desirable to test the point. For the purpose the hydrogen ion concentration of the perfusate, with and without dorsal nerve root stimulation, was determined in a preparation which had immediately before such a test, and immediately after, given a good effect on the heart. The quinhydrone capillary electrode method was used. The following measurements were obtained:

pH of perfusing fluid, 7·3.
pH of perfusate with dorsal nerve root stimulation, 7·0.
pH of perfusate without stimulation, 7·0.

The effect is therefore not due to changed hydrogen ion concentration.

Since vaso-dilatation is the only known effect of dorsal nerve root stimulation, and since there is good evidence for the chemical transmission of the effects of such stimulation (Lewis and Marvin, 1927), one cannot but suspect that the cardio-depressor substance is identical with this chemical transmitter.

Of the known vaso-dilator substances it is possible to consider only histamine and acetylcholine. For the frog, however, histamine must also be excluded, since it does not cause dilatation in that animal, nor is there any evidence of its occurrence in the frog's skin (Grant and Jones, 1929). On the other hand a depressor action on the heart, abolished by atropine, and augmented by eserine is a combination of features which it presents in common with acetylcholine.

Whether the effects are due to the action of antidromically stimulated sensory fibres, or efferent parasympathetic fibres stated to occur in the dorsal roots (Kure, 1928, 1930) has not been determined.

SUMMARY.

Dorsal nerve root stimulation releases in the skin of the frog a substance which has in common with acetylcholine the capacity of depressing the heart, the effect being abolished by atropine and augmented by eserine.

I am much indebted to Mr. H. Ennor for technical assistance.

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THE LYSOGENICITY OF COLIFORM BACILLI

by

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While several workers (Gildemeister and Herzberg, 1924; Lisbonne and Carrière, 1922) have reported that bacteriophages active against dysentery cultures can be obtained from coliform strains, there has apparently been no attempt to classify these phages or to study as Burnet (1932) has done for the Salmonella group, the relationship between the nature of the culture producing phage and that of the indicator strain detecting its presence. This paper records the results obtained when 119 coliform organisms of human origin were investigated for lysogenicity.

As a routine, the cultures were grown for 24 hours in pH 8·0 broth, were filtered, and the filtrates were tested on 1 p.c. agar plates spread with a series of indicator strains. Various cultures (including strains of Flexner and Shiga dysentery both smooth and rough, *B. suipestifer*, *B. coli anaerogenes*, *B. coli communior*, *B. sanguinarium*, etc.) were tested, but finally a series of six strains, all non-lysogenic themselves, was chosen. If a phage were present in the primary filtrate, as shown by the presence of plaques on an indicator strain, it was isolated by the usual methods.

The cultures examined fell into three groups:

i. Typical coliform organisms (82).

ii. Atypical B. coli strains consisting of (a) late lactose fermenters including *B. coli mutabile* (6); (b) Strains giving irregular fermentation results, producing acid and gas in some sugars and acid only in others (3).

iii. Anaerogenes strains (a) *B. dispar* and *B. coli anaerogenes* (12); (b) *B. alkalescens* (3); (c) Sonne dysentery (13).

Table 1 shows the results obtained with the 119 cultures (the lysogenic *B. neapolitanus* strain coli 88 described by Gildemeister and Herzberg (1) is included).

TABLE 1.

Lysogenicity of Coliform Cultures.

Species.	Source of strains. Urine. Lysogenic.	Urine. Non-lysogenic.	Faeces. Lysogenic.	Faeces. Non-lysogenic.	Other human sources. Lysogenic.	Other human sources. Non-lysogenic.	Total Lysogenic.	Total Non-lysogenic.
i. *B. coli*								
B. coli communis	7 (1 weak)	21	6	5	2	0	15 = 36·6% (1 weak)	26
B. coli communior	2	3	0	3			2 = 25%	6
B. neapolitanus	2	11	2	3	1*	0	5 = 26·3%	14
B. acidi lactici	2 (2 weak)	5	0	7			2 = 14·3% (2 weak)	12
ii. *Atypical B. coli*	3 (1 weak)	2	3 (2 weak)	1			6 = 66·7% (3 weak)	3
iii. *B. anaerogenes*	4 (1 weak)	8	3	13			7 = 27% (1 weak)	21

* B. coli 88 of Gildemeister and Herzberg.

Thus, of 119 cultures, 37 or 31·1 p.c. gave phages in the primary filtrate, and in several cases two or more apparently distinct phages were isolated from the same filtrate, the total number of phages obtained being 52. Gildemeister and Herzberger (1924) found that 5 out of 9 coliform strains gave a phage active against a smooth Shiga culture, but, in our experience, a rough Flexner VR culture was of much more value in detecting lysogenicity. Of the 52 phages obtained from the coliform cultures 38 (including the coli 88 phage) were active against VR and only 8 against smooth or rough Shiga strains. A fair proportion (13) acted on the rough *B. sanguinarium*, 398 R, but very few were active against smooth *B sanguinarium* or Flexner dysentery cultures or against the typical *B coli communior* strain, KR, whose phage reactions have been described previously (Burnet and McKie, 1933). None of the filtrates tested gave confluent or even semi-confluent lysis on agar with the indicator strains, the majority giving a few plaques per drop. Further, all phages obtained were weak, and none gave complete clearing in broth or on agar. The plaques were usually small with central secondary growth and were never greater than 1·5 mm. in diameter on 1 p.c. agar. Of the 52 phages 15 were particularly weak and were lost in passage. The remaining 37 were made up of:

1. 28 active against and grown with Flexner VR.
2. 3 grown on coli R, weakly active on VR but growing poorly at its expense.
3. 3 active on 398R, inactive on VR.
4. 3 weak specific smooth phages active on 398S, Shiga S, Shiga S and YS respectively.

A point of some interest was that the filtrates of many cultures had an inhibitory action on the growth of one or more indicator strains, although they contained no phage. Filtrates that undiluted gave clear lysis on agar possessed little or no action when diluted 1 in 10, although occasionally with this dilution there were weak uneven patches where growth appeared to have been somewhat inhibited. The inhibitory power of the filtrate was completely non-transmissible, being quite lost on passage, and our results were very similar to those recorded by Gratia (1932) for his coli V. strain. Resistant colonies were of common occurence but were not studied in detail. Most frequently the filtrates were inhibitory for the rough strain Flexner VR and for a rough *B. coli communior*, but occasionally other strains were sensitive. Filtrates of non-gas-producing strains appeared to possess the property of inhibition more often than those of the other types studied (Table 2).

TABLE 2.

		Number studied.	Number inhibitory.
Group i.	Typical coliform organisms.	82	6 = 7%
Group ii.	Atypical B. coli strains	9	1 = 11%
Group iii.	Anaerogenes strains	28	10 = 35·7%

Further investigation was confined to the 28 phages active on VR, the group being studied from the standpoint of its cross-resistance reactions and serology.

1. *Grouping by Cross-resistance.*

Members of the group were tested on the standard resistants to C and D phages of the coli-dysentery group (Burnet, 1930), and were typed into C, D, and N phages according to the scheme shown in Table 3.

TABLE 3.

	Normal dysentery.		R. dysentery resistant to	
Phage type.	S	R	C phage.	D phage.
Type C	+	+	—	+
D	+ or —	+	+	—
N	+ or —	+	+	+

In addition rough resistants to 27 of the 28 phages were obtained without difficulty from VR. The results obtained when each phage was tested on its homologous resistant and the heterologous resistants are shown in abbreviated form in Table 4.

TABLE 4.

	Serial number.	Group and resistance type.	Action of phages on: YS Smooth Flexner dysentery.	VR Rough Flexner dysentery.	398R Rough *B. sanguinarium*.	VR Resistant to: Group 1	Group 1a)	Group 1b)	Group 2	Group 2a)	Group 3	Group 4	Neutralization by Anti-phage sera. L34	L2	L15
1	4 10 16 19 23 N N N N N		—	+	+	—	±	—	+	—	—	+	—	—	—
1a)	5 15 D D		+	+	+	±	—	—	+	+	+	+	—	+	+
1b)	28 N		—	+	+	+	+	—	+	—	—	+	—	+	+
2	1 3 40 41 N N N N		—	+	—	+	+	±	—	—	+	+	+		
2a)	6 12 31 34 37 C C N N N		—	+	—	+	+	+	+	—	±	+	+	—	
3	2 39 33 D D D		+	+	±	+	+	±	+	+	—	+		+	+
4H	9 11 18 47 14 22 29 C88 D N N N D D D D		—	+	—	+	+	±	+	+	±	±	—	—	—
															Variable

Signs used: + Consistent lysis. — Consistent failure to lyse. ± Irregular results.

While the results obtained from the cross resistance tests with these weak phages are not so clear-cut as those obtained when typical active phages are tested, there is an obvious tendency for them to fall into groups and in general the members of each sub-group have the same plaque type (for instance, all the phages of Group 1 have exactly similar very weak hazy plaques with the same range of plaque size, while the plaques of all four phages of Group 2 are very small and clear with a refractile edge).

2. *Grouping by Antiphage Sera.*

The application of serological methods to the classification of these phages presented considerable difficulties. In the first place the filtrates obtained were nearly all of low titre and proved very lacking in antigenicity. One phage L34 gave a satisfactory serum after immunization of a rabbit by the ordinary method with a filtrate from a partially lysed culture, but none of the others tested gave any detectable activity by this method. Two other antiphage sera were obtained (against L2 and L15) by immunizing with unfiltered semilysed cultures sterilized by formalin, but even this method failed with representatives of Group 1.

A second difficulty was the occurrence of partial neutralization when some of these phages were treated either with a heterologous antiphage serum or with

any R dysentery antiserum. Apparently the antibacterial components of the sera produced sufficient antibody coating of the bacteria on which the phages were tested to hinder the attack of these very weak phages.

The results obtained are included in Table 4. The three sera used delimit two distinct serological groups. The very weak anti-L4 serum significantly inhibited L16 and L19 but was practically without effect on the homologous phage and L10 and L23. It is probable, however, that all five of the phages in Group 1 would fall into one serological group if a sufficiently potent serum were available. Of the final heterogeneous group (H) L9, L14, L22, L47, and C88 were neutralized by anti-L15 serum and usually by L2 antiserum. L11 and L18 were not.

Representative phages of this series were tested with antisera made against each of Burnet's small plaque types of coli-dysentery phages (Nos. 9, 10, and 11) without showing any significant neutralization.

Relation of Lysogenic Phages to Other Phages Acting on Rough Dysentery Strains.

As far as the work has gone the results suggest that none of these weak phages fall into any of the groups of active dysentery phages studied in detail and classified by Burnet, 1933. Typical phages from his small plaque groups 7 (C 18), 9 (C 33), and 11 (D4, C16, H, J, K) were tested on 15 representative resistant strains, and in every case lysis was complete. As will be seen from Table 4 when the strains were tested on standard dysentery resistant strains, the results indicated that a majority are N phages in the terminology of Burnet and McKie, but in one serological group (2 and 2a) two phages show C type (major Group 1) reactions, while other members of the group are N phages but with a tendency to the C type reactions. The other serological group of phages contains mostly D type phages probably representing another type of major Group 2 phages.

Judged from the type of plaque, small and sharp-edged, and the cross resistance and serological results, it appears legitimate to place the phages of the serological group exemplified by L34 in the table published by Burnet (1933) as belonging to major resistance Group 1 and plaque type *V*.

Relationship between the Type of Phage produced and the Lysogenic Strain.

The biochemical activities of the phage-producing strain and the type of phage produced could not be definitely co-ordinated. Of the nine strains of Group 2 and 2a completely neutralized by L34 serum, one was from a Sonne strain, five from *B. coli communis*, one from *B. coli communior* and two from *B. dispar*. Further, the same type of coliform bacillus (classified by its action on saccharose, salicin, and lactose (Topley and Wilson, 1928)) produces phages of different type, e.g., four of the five weak phages of Group 1 are from *B. coli com-*

munis strains, and so are the four clear small plaque phages of Group 2 which are completely neutralized by L34 serum.

The eleven phages neutralized by L15 antiserum were from *B. coli communis* (4), *B. neapolitanus* (3), *B. coli communior* (1), *B. coli anaerogenes* (2), and Sonne dysentery (1).

Table 1 shows the percentages of phages given by the various types of coliform bacilli. Excluding the heterogeneous atypical cultures, *B. coli communis* strains appear to be most frequently lysogenic, while *B. acidi lactici* (which have weak powers of fermenting carbohydrates) are least lysogenic of all the types studied.

On account of the absence of available data and the known extreme serological heterogeneity of coliform bacilli no attempt could be made to determine whether the strains producing similar phages showed any common serological behaviour.

DISCUSSION.

These weak phages have proved on the whole very unsatisfactory material for study, but they nevertheless represent probably the most frequently occurring bacteriophages in nature, and must be considered in any attempt to assess the biological status of this group of micro-organisms. Probably because of the great heterogeneity of the organisms grouped under the general term B coli, no indications of a consistent type of lysogenesis such as was found in certain of the Salmonella races by Burnet have been observed. In regard to the nature of the phages themselves it seems clear that apart from their low grade of activity they do not differ fundamentally from the active phages which have been studied. Most of them fit into one or other of the two major resistance groups previously defined, and possess distinct serological individuality. The complexity of the cross resistance tests is probably to be related to the weakness of their lytic activity, specific resistance being readily evoked and requiring only minor modifications within the bacteria.

SUMMARY.

1. One hundred and nineteen strains of coliform bacteria of human origin have been studied in regard to the presence and type of lysogenicity.

2. Phages were detected in filtrates from 37 (31·1 p.c.) strains, and in several cases two or more apparently distinct phages were isolated from the same filtrate.

3. The great majority of the phages were detected by the use of a rough Flexner strain as indicator organism; a considerable proportion also acted on *B. sanguinarium* (rough), but practically none on rough coli or Shiga strains or on any of the corresponding smooth forms.

4. All the phages obtained were weak and a number were lost in attempts

at passage, but 28 were sufficiently active to allow of their classification by the methods used in previous work.

5. The filfrates of a number of non-lysogenic cultures were strongly inhibitory for some of the indicator strains, especially for rough dysentery cultures.

6. The results obtained from the study of the 28 phages by cross resistance and serological tests suggest that they are distinct from any of the active dysentery phages previously studied, but that a majority of them fall into three fairly distinct groups.

7. The cross resistance tests show many anomalies, and are difficult to co-ordinate with the relatively clear indications obtained by the same methods with typical active phages.

8. There is no evident co-ordination between the type of phage produced and the biochemical activities of the producing strain.

ACKNOWLEDGMENT.

I am indebted to Dr. F. M. Burnet for suggesting this investigation, and for criticism and help in presenting the results.

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THE PERIPHERAL ACTION OF AUSTRALIAN SNAKE VENOMS

4. ACTION ON SENSORY NERVE ENDINGS IN FROGS.

by

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(Submitted for publication 21st July, 1934.)

In earlier papers in this series (1932) it has been shown that Australian snake venoms have a paralysant action upon motor endings in muscle in both frogs and mammals. The present communication is concerned with their action upon sensory endings in the frog.

Method. The frogs used were *Hyla aurea*. The venoms were tested upon the dorsal skin preparation used by Adrian (1926) and Bronk (1929), and the peroneal toe muscle preparation described by Matthews (1931a).

In testing tactile endings, four to six dorsal cutaneous nerves were prepared and tested in succession after the endings connected with the nerve first tested were paralysed. The nerves were placed one at a time in a moist chamber across cotton thread electrodes of the type used by Adrian. The excised dorsal skin was placed, with the external surface uppermost, on the thin cork roof of the vulcanite moist chamber, and the nerve entered the chamber through a slit. The nerves awaiting test were kept moist between the skin and the cork. Tactile stimuli were applied by light touch with the tip of a primary wing feather of a fowl. The skin was kept wet throughout the experiment with venom solution of the strength under test.

In the study of muscle spindles, the apparatus used was that devised by Matthews (1931), which was made for me by my colleague, Mr. E. Hughes. No attempt was made to secure single ending preparations, since in pharmacological experiments it is advantageous to use preparations in which many end organs are functioning. Under these conditions each experiment tests the most resistant of a group of organs of varying susceptibility. The tension on tendons was usually applied by a five gramme weight at the end of a lever, whose motion was controlled by a plunger in a dashpot containing heavy oil. The physiological saline solution, or the snake venom dissolved in this solution in the small vulcanite trough which contained the preparation, was changed every five or ten minutes throughout the experiment. This involved the use of large volumes of venom solution for each experiment. In the period between observations the nerves were kept immersed in the solution in the trough.

The experiments recorded here were carried out between March 12th and July 5th, 1934. The frogs used had not been longer than 14 days in captivity. The experiments were made in a basement room, the temperature of which during this period ranged from 16° to 20°C. Most of the time it was 18° or 19°C. When the temperature rose above 20°C. the physiological saline solution and the venom solutions under test were cooled to temperatures ranging from

3°C. to 12°C., and kept in thermos flasks. In this way, by frequent changes, it was possible to keep the preparations at reasonably low temperatures.

The physiological saline solution was made up freshly each day. It contained NaCl 0·65 p.c., KCl 0·014 p.c., $CaCl_2$ 0·024 p.c., and $NaHCO_3$ 0·02 p.c. Various buffers other than bicarbonate were tried, but this proved most satisfactory. At temperatures not exceeding 19°C., skin preparations kept moistened with this solution, and peroneal nerve toe muscle preparations in which the solution was changed at frequent intervals, remained in excellent condition for eight or ten hours.

The amplifying system used was very similar to the resistance capacity coupled amplifier at present used by Adrian and by Matthews. The first two valves (Mullard 904 V), and the batteries supplying them, were enclosed in an earthed, air-tight metal box, lined with celotex. The capacities used in this amplifier were 0·1 micro farads. The output could be connected either with a loud speaker amplifier, or with amplifier No. 1 of the Matthews oscillograph through a volume control, so arranged that fractions from $\frac{1}{5}$ to $\frac{1}{50}$ could be further amplified. A rather elaborate system of screening around the preparation was necessary to eliminate a number of sources of electrical disturbance.

The Action of Venoms on Tactile Endings of the Skin.

The results of 22 experiments with various venoms are summarized in Table 1, in which are given the longest and shortest observed times necessary for a given concentration of venom to cause extinction of the response to tactile stimuli. The experiments were carried out at 18° or 19° C. and in nearly all of them 3 or 4 nerves were tested. Whereas skin kept moist with physiological saline solution remained in good condition for eight hours or more, giving a good tactile response at $\frac{1}{50}$ amplification, the venoms caused within variable times progressive weakening of the response, so that it could be only heard or optically recorded if increasingly great amplification was applied to the action currents produced in the nerve. The area of skin from which the response could be elicited often diminished in size before total failure to respond, the more peripheral endings being first paralysed. Finally all the endings were paralysed and no action current could be detected following the application of a stimulus. At no stage were the endings stimulated by the venom so as to produce spontaneous action currents. In these experiments the nerves were also exposed to the action of the venoms, but in control experiments in which the skin was kept wet with saline and the lower part of the nerves repeatedly wetted for 7 or 8 hours with venoms in a concentration of 1 : 1,000, this had no effect on the action currents in the nerve in response to stimulation of the skin, if the venom were kept away from the skin and the nerve endings in it.

TABLE I.

The Action of Snake Venoms on the Tactile Skin Endings of HYLA AUREA.

Snake Venom.	Concentration.	Time to paralysis of endings.
Indian cobra (*Naia naia*)	1: 100,000	3½ hours.
	1: 10,000	1 hour 15 minutes to 1 hour 48 minutes.
Black snake (*Pseudechis porphyriacus*)	1: 100,000	5 hours 43 minutes.
	1: 10,000	2 hours 18 minutes to 2 hours 53 minutes.

TABLE I. (continued)

The Action of Snake Venoms on the Tactile Skin Endings of HYLA AUREA.

Snake Venom.	Concentration.	Time to paralysis of endings.
Copperhead	1 : 10,000	4 to 5 hours.
(*Denisonia superba*)	1 : 2,500	2 hours 53 minutes to 3 hours 43 minutes.
Death adder	1 : 2,000	2 hours 45 minutes to more than 3 hours 8 minutes.
(*Acanthophis antarcticus*)	1 : 1,000	2 hours 8 minutes to 4 hours 20 minutes.
Tiger snake	1 : 2,000	2 hours 20 minutes to 4 hours 23 minutes.
(*Notechis scutatus*)	1 : 1,000	1 hour 53 minutes to 2 hours 40 minutes.
Black tiger snake	1 : 1,000	2 hours 13 minutes to 3 hours 8 minutes.
(*Notechis scutatus* var. *niger*)		

These tactile endings appear to be somewhat more resistant to the action of venoms than the motor endings in the isolated gastrocnemius sciatic preparation Kellaway and Holden (1932). The order of activity of the venoms is, however, much the same as that determined for paralysis of motor endings. The venom of the Indian cobra *(Naia naia)* is more potent than any of the Australian venoms, and that of the Australian black snake *(Pseudechis porphyriacus)* is the most active of the Australian venoms tested. The venom of the copperhead *(Denisonia superba)* is next in activity, and the remaining three venoms are active only in high concentrations.

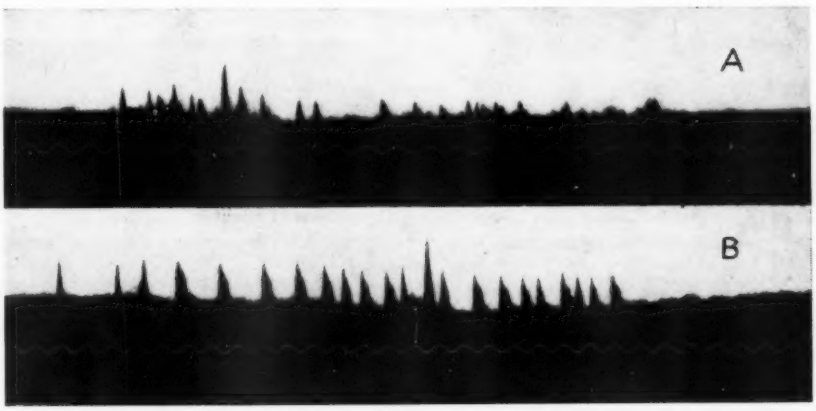

Figure 1. Action currents in dorsal cutaneous nerve of frog in response to light touch on tactile sensory endings. A at $\frac{1}{50}$, and B at $\frac{1}{20}$ amplification. Records read from right to left. Time in $\frac{1}{100}$ second.

In Figure 1a are seen the action currents at $\frac{1}{50}$ amplification produced by dragging the tip of a feather with the pressure of its own weight across the area of normal skin supplied by the nerve. The time is given by a tuning fork, 100 vibrations per second. The whole response lasts less than $\frac{1}{5}$ second. A rather

longer response is seen in Figure 1b at $\frac{1}{20}$ amplification. The responses obtained from preparations treated with venom before the endings are paralysed may be fewer in number, and higher amplification is necessary to record them.

The Action of Venom on Muscle Spindles in HYLA AUREA.

There were two groups of these experiments. In the first, carried out during a hot spell at the end of March, the laboratory temperature ranged from 19° to 22°C. It was found, however, that control preparations survived for 8 or more hours without impairment of the response if the temperature of the physiological saline in which they were immersed were cooled to temperatures below 12°C. and the saline were changed at 5 minute intervals.

The results of this first series of experiments are summarized in Table 2.

TABLE 2.

The Action of Venom on Muscle Spindles in HYLA AUREA *in Autumn. Room Temperature 19° to 22°C.*

Venom.	Concentration.	Temperature of physiological saline.	Time to cessation of all activity.
Cobra (*Naia naia*)	1: 10,000	6·5°C.	4 hours 19 minutes.
	1: 2,500	3°C.	After three hours only spontaneous firing.
		4·5°C.	2½ hours.
		6·5°C.	3 hours 6 minutes.
		11·5°C.	23 minutes.
Black snake (*Pseudechis porphyriacus*)	1: 5,000	6·5°C.	6 hours.
	1: 2,500	7°C.	1 hour 10 minutes.
		7°C.	4½ hours.
Copperhead (*Denisonia superba*)	1: 10,000	12·5°C.	3½ hours.
	1: 5,000	6°C.	4 hours 10 minutes.
		8·5°C.	Only spontaneous firing after 3¾ hours.
	1: 2,500	8·5°C.	1 hour 37 minutes.
		9°C.	25 minutes.
Death adder (*Acanthophis antarcticus*)	1: 2,500	7·5°C.	6½ hours.
		9°C.	Only spontaneous firing after 4½ hours.

In all these experiments the motor endings in the preparation were paralysed before the sensory endings. Since there was a negative bias of 1·5 volts on the grid of the first valve, contraction of the muscles of the toe occurred when the nerve was placed across or taken down from the electrodes so long as the motor endings remained intact. This response ceased long before that of the muscle spindles. Contraction of the muscle was often followed by strong action currents in the nerve due to the discharge of many muscle spindles presumably stimulated by the contraction (Matthews 1931b). This phenomenon was much more frequently observed in the later experiments carried out in mid-winter at rather lower room temperatures 15·5° to 17°C. The firing died away after a few minutes, and it was then easy to test the effect of tension upon a tendon.

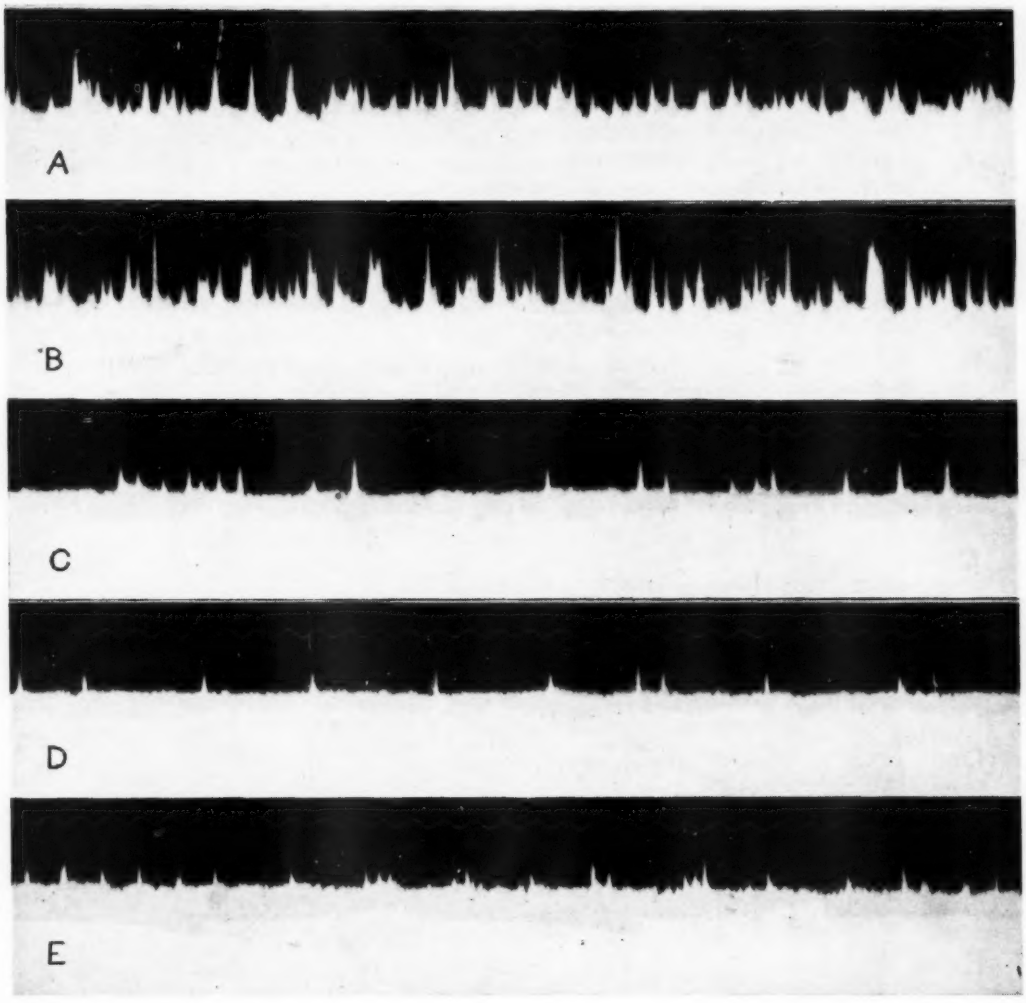

Figure 2. Action currents in peroneal nerve from toe muscle preparation. A at $\frac{1}{20}$ amplification immediately after loading the right tendon with a 5 gm. weight; B at $\frac{1}{10}$ amplification with a similar load on the tendons after $1\frac{2}{3}$ hours in 1: 2,500 death adder venom; C at $\frac{1}{10}$ amplification showing spontaneous firing after two hours in venom; D at $\frac{1}{10}$ amplification tendon load 5 gm. after $3\frac{1}{2}$ hours in venom; E at $\frac{1}{7}$ amplification with the same load after 4 hours in venom. Records read from left to right. Time in $\frac{1}{100}$ second.

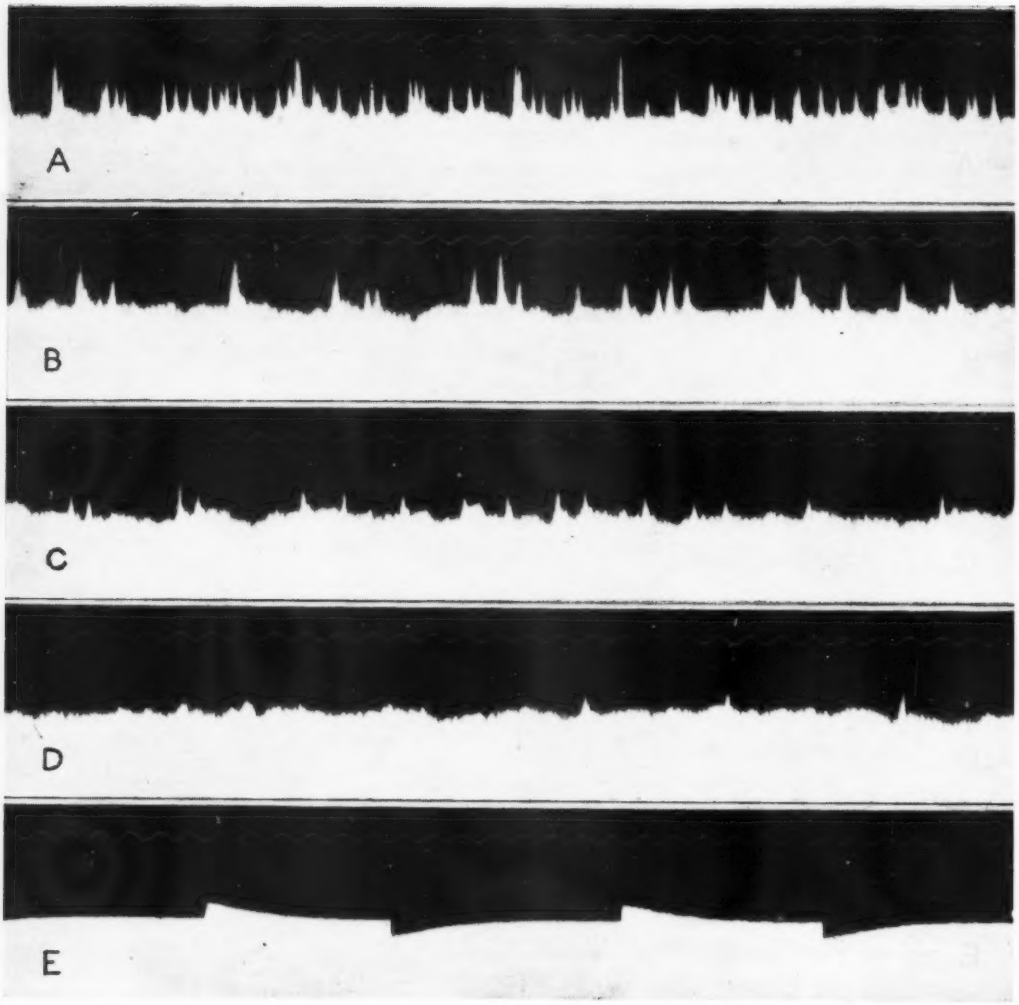

Figure 3. Action currents in peroneal nerve from a toe muscle preparation. All records at $\frac{1}{10}$ amplification within 30 seconds after loading the right tendon with a 5 gm. weight: A before venom, B after 20 minutes in 1: 2,500 cobra venom, C after 66 minutes in venom, D after 2 hours in venom. E calibration 10 micro volts at $\frac{1}{10}$ amplification. Records read from left to right. Time in $\frac{1}{100}$ second.

In nearly all these early experiments a series of photographic records was made (Figs. 2 and 3). Before total paralysis of the nerve endings, there was first a period of diminution of the intensity of the response. In the late stages, higher amplification was often necessary to obtain satisfactory records. The number of endings firing in response to a stimulus was reduced ultimately to 1 or 2, the remainder being paralysed. Finally, intermittent spontaneous firing of 1 or 2 endings was the only evidence of activity, and no detectable increase in frequency was observed to follow tension applied to the muscle tendon. This period of spontaneous firing without added response to stimulus lasted for variable periods, and in experiments with weak venom solutions often for an hour or two before the total extinction of all activity.

In Table 2, despite variation in the resistance of preparations dependent partly upon individual variation in the animals and partly upon variable degrees of trauma in setting up the preparations, there is clear evidence of the influence of temperature upon the rate at which the activity of the endings is extinguished by venom.

The second series of experiments was carried out between June 11th and July 5th, and stronger solutions of venom, all of which were used at room temperature, were needed to obtain end results within a reasonable period of time. These results are summarized in Table 3.

TABLE 3.

The Action of Venoms on Muscle Spindles of HYLA AUREA *in Midwinter.*

Venom.	Concentration.	Room Temperature.	Time to cessation of all activity.
Indian cobra (*Naia naia*)	1: 2,500	18°C.	2½ hours.
		19°C.	4 hours 50 minutes.
	1: 1,000	17°C.	2 hours.
		17°C.	30 minutes.
Black snake (*Pseudechis porphyriacus*)	1: 2,500	16°C.	Spontaneous firing not quite abolished after 5¾ hours.
		19°C.	4¾ hours.
	1: 2,000	17°C.*	Endings not paralysed in 6 hours.
		17°C.	2¼ hours.
	1: 1,000	16°C.	3¾ hours.
Copperhead (*Denisonia superba*)	1: 2,500	18°C.	2½ hours.
	1: 2,000	15·5°C.	5¾ hours.
		17·5°C.	2 hours.
	1: 1,000	14°C.	3¼ hours.
Death adder (*Acanthophis antarcticus*)	1: 1,000	16°C.	8½ hours.
		16·5°C.	5⅔ hours.
Tiger snake (*Notechis scutatis*)	1: 1,000	17°C.	7½ hours.
		19°C.	5⅓ hours.
		19°C.	4 hours 10 minutes.
Black tiger snake (*Notechis scutatis* var. *niger*)	1: 1,000	16°C.	5¼ hours.

* Venom solution cooled to 3°C.

In this second series of experiments the muscle spindles are much less susceptible to the action of venom than the earlier series, if we can assume, as I think we may, that the temperature of the preparations in the first series approached that of the venom solutions in which they were bathed. If this were the case, the results point to seasonal variation in the frogs, winter frogs being less susceptible to the action of venom. The resistance of the frogs in the late autumn experiments may have been impaired by keeping them a few days in captivity during the abnormally hot weather at temperatures round 20°C. before they were used. This seems rather unlikely since before the addition of venom the preparations were washed repeatedly with cold physiological saline and in control experiments remained in excellent condition for many hours.

The alternative explanation of this difference of behaviour in the two groups of preparations is that in the autumn experiments the mean temperature of the preparations was much higher than in the winter experiments despite the cooling effect of frequent changes of cold venom solution.

In all these experiments the nerve was immersed in the venom solutions. To exclude the possibility that extinction of the action currents in response to stimuli was due to injury to the nerve, a number of experiments of the following type were made with the more active venoms.

Experiment 1. July 3rd. Room temperature 17°C.

10 a.m. Peroneal nerve preparation set up.

10.18 a.m. Using the loud speaker, audible responses to tension of both tendons were obtained at $\frac{1}{50}$ amplification. The responses were loud at $\frac{1}{20}$, and there was some spontaneous firing.

10.20 a.m. The preparation was placed in one compartment of a small petri dish lined with wax and divided into compartments. The nerve passed through a small vertical slit packed with vaseline into the second compartment. This was filled with cobra venom solution 1: 1,000, the preparation in the other compartment being covered with physiological saline which was changed every 10 minutes throughout the day.

4.25 p.m. Audible responses at $\frac{1}{50}$ amplification to tension of both tendons. These were strong at $\frac{1}{20}$, and there was some spontaneous firing. No change had taken place in the action currents in 6 hours during which the nerve had been immersed in strong venom solution.

4.27 p.m. Preparation and nerve placed in cobra venom solution 1: 1,000.

5.12 p.m. Tension of the left tendon gave a good response at $\frac{1}{20}$ amplification, but of the right only at $\frac{1}{10}$ amplification. Some spontaneous firing was audible at $\frac{1}{20}$.

5.23 p.m. From the right side there was no response. From the left there was still a good response at $\frac{1}{10}$ amplification. There was also some spontaneous firing.

5.58 p.m. Spontaneous firing had ceased. From the left tendon, weighted with 5 gm., a single ending response was obtained at $\frac{1}{20}$ amplification.

6.14 p.m. The single ending response had now diminished in frequency at $\frac{1}{10}$ amplification, and irregular spontaneous firing was present.

6.21 p.m. No response to stimulus—slow, irregular, spontaneous firing.

6.28 p.m. All activity extinguished.

All the snake venoms tested, though they have no appreciable effect upon the nerves, can paralyse sensory endings *in vitro*. In the case of the venoms of the

tiger snake and death adder this action upon nerve endings is relatively feeble, high concentrations being necessary for its demonstration in reasonably short times. Even in the most active of the venoms it is extremely feeble compared with that of a good local anaesthetic. Percaine, for example, in a concentration of 1 : 100,000 completely paralyses endings in the skin in 12 minutes at 15°C. Incidentally this method offers a good objective test for the comparison of local anaesthetics, and mammalian skin cutaneous nerve preparations might well be used for this purpose.

It was of some interest to ascertain whether this paralysis of sensory endings resulted when the intact frog was poisoned with venom. For this purpose venoms were selected for test which contained no thrombin, since those containing thrombin have a rapidly lethal action in the frog, causing intravascular coagulation, and would kill too quickly for paralysis of the sensory endings to occur. It was also possible that some frogs would be killed too rapidly by other lethal actions, such for example as the effect of cobra venom upon the heart muscle. Experiments were carried out with the venoms of the Indian cobra, of the Australian death adder and copperhead and with a sample of tiger snake venom freed from thrombin by Holden's (1932) method. The venoms were injected in a concentration of 1 or 2 mg. per c.c. Immediately after the animal was dead, or in some cases after pithing when motor paralysis was complete, skin and toe muscle peroneal nerve preparations were set up and tested.

With cobra venom, of four frogs which received 4 mg. per 100 gm. subcutaneously, three died in 2, 2½, and 2¾ hours, and one, which was paralysed in four hours, was pithed and tested after five hours. The skin endings were normal in all except the frog which survived longest, in which they gave no response. The muscle spindles were not tested in the first frog, and were paralysed in two of the remaining three.

With death adder venom, three frogs received 5 mg. per 100 gm. and died in 5½, 6½, and 7½ hours. In that which survived longest the skin and muscle endings were unaffected, but in the other two both were paralysed. In two frogs which received 4 mg. per 100 gm. one died in 3½ hours, and one was tested after pithing the frog three hours after the injection of venom. In both these the skin endings were paralysed. The muscle endings were only tested in the pithed frog, and these were unimpaired. One frog was injected with tiger venom fraction 4 mg. per 100 gm. This was pithed after 24 hours when there was complete motor paralysis. The tactile endings in the skin were paralysed but the muscle spindles still functioned well. A frog injected with copperhead venom, 4 mg. per 100 gm., died in 21½ hours, and both skin and muscle endings gave no response to test.

It appears then that the venoms have a rather irregular action on sensory endings in the envenomed animal. The clinical picture of poisoning in man by some of these venoms suggests that some action would be demonstrable since parasthesia is of frequent occurrence.

It is of some interest to know whether curari, which has so potent an action

on motor endings, can also paralyse sensory endings. In two experiments in a concentration of 1 : 1,000 at 16°C. and 16·5°C. it caused complete paralysis of frog muscle spindles in $3\frac{1}{4}$ and in 4 hours. In a third experiment in a concentration 1 : 2,000 there was very little impairment of response in five hours. This sample of curari in a concentration of 1 : 100,000 paralyses the motor endings of *Hyla aurea* in between $2\frac{1}{4}$ and $4\frac{3}{4}$ hours (Kellaway and Holden, 1932), and in the present experiments the motor endings were paralysed in less than an hour. Curari appears to have but little effect on the tactile endings in skin. In one experiment in a concentration of 1 : 1,000 no effect was observed in four hours, but in another the endings were paralysed in 7 hours at 16° C.

As was to be anticipated from these results in the intact frog, the effects on sensory endings are completely overshadowed by the predominantly paralytic effect on motor endings when minimal effective doses of curari are used. For this species of frog the curarizing dose of this sample of the drug is 2 mg. per 100 gm. (Kellaway and Holden, 1932). Three frogs were given 4 mg. and were pithed $4\frac{1}{2}$, 6, and 17 hours after the injection, during which time they remained completely immobile. Tests with these frogs of the sensory endings in skin and muscle showed no sign of any impairment in their responses to stimuli.

CONCLUSIONS.

1. The snake venoms tested have a paralysant action *in vitro* on sensory nerve endings in *Hyla aurea* though this is less powerful than their action upon motor endings in this species.

2. The arrangement of the venoms in the order of their potency is the same for both sensory and motor nerve endings.

3. Curari paralyzes sensory endings *in vitro*, but this action is overshadowed by its much more powerful effect on motor endings.

4. The method used here, when applied to mammalian endings, is suggested for comparison of the activity of local anaesthetics.

ACKNOWLEDGMENTS.

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OBSERVATIONS ON THE SPECIFIC ROTATION OF CRYSTALLINE EDESTIN

by

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Following on the experiments performed in this laboratory on the effect of denaturation on the specific rotation of hen ovalbumin and of the proteins of mammalian sera (Holden and Freeman, 1930), an attempt was made to extend the work to include similar studies on a representative of the crystalline vegetable globulins. Edestin was the protein selected.

Estimations of the specific rotation of native crystalline edestin from hemp seed have been made by Osborne and Harris (1903), Chittenden and Mendel (1894), and by Alexander (1896). The latter also records observations of the specific rotations of solutions denatured by the addition of dilute acids and alkalis.

As will be seen from the paper the major portion of the work proved to be the isolation of edestin in such a manner that specimens prepared at different times and from different samples of hempseed should have a uniform optical activity. Although in the author's hands the ordinary methods of preparation of crystalline hen ovalbumin yield specimens which differ in specific rotation only by very small amounts, specimens of edestin were found to vary by several degrees in rotation following apparently slight variations in the method of preparation employed.

EXPERIMENTAL.

The oil was removed from ground-up hemp seed by several extractions with ice cold ether or petroleum ether. After removal of the solvent by drying in air at the temperature of the laboratory, the hemp seed was extracted with 10 p.c. sodium chloride solution for three-quarters to one hour at temperatures ranging from 15°C. to 60°C. Approximately 500 c.c. of sodium chloride solution were used for every 100 gm. hemp seed. After rapid filtration by means of a screw press, water at 40°C. was added to the warm solution until it became turbid. On cooling slowly, well developed crystals separated out. After washing the edestin with distilled water in the centrifuge, it was recrystallized from 10 p.c. salt solution, warmed to 40°C., filtered and diluted with water at 40°C., as in the first crystallization. The crystals were dissolved in 10 p.c. sodium chloride solution, and kept in the refrigerator. The solutions were very slightly opalescent, and observed through the depth of 1 dm. they had a pale straw colour. To avoid all

unnecessary handling, the final drying with alcohol and ether employed by other workers was dispensed with, except when it was desired to study the effect of this process on the rotation.

Optical rotations were read by a Hilger polarimeter using the mercury green line.

Measurements of hydrogen ion concentration were made with a quinhydrone and calomel electrode.

Estimates of protein content were made by Devoto's method of coagulation in saturated ammonium sulphate solution in the earlier experiments. Difficulty was found in prevention of solution of the protein in water, after removal of most of the ammonium sulphate. It was better to coagulate the protein on a boiling water bath after dilution of the salt to 1–2 p.c. by water, keeping the pH of the solution at 6·5 to 7·0. In later experiments protein was determined by Kjeldahl's method, using the factor 5·35 to convert the nitrogen to protein percentage. This figure was determined by Osborne (1892) and by Chittenden and Mendel (1894). In addition it was confirmed on three of the specimens prepared in this research, the protein content of which had been estimated by the coagulation method.

Constant values could not be obtained for the specific rotation. They varied from —50°, which corresponds approximately to Osborne's figure of —41·4° for the D line, to—59°, without showing obvious effects of denaturation. The solutions contained no suspended precipitate. The figures obtained are shown in Table 1. Numerous experiments of each type shown there were made.

The data in Table 1 show that edestin is an unstable substance. The most rapid preparations yield edestin with the lowest specific rotation —50° to —53°. The pH of the extractions and solutions in 10 p.c. sodium chloride was between 6·3 and 7·0 in all the ordinary preparations. Changes in this range of pH do not affect the specific rotation. Experiments have been made adding small amounts of acid and alkali to give pH's as acid as 6·1 and as alkaline as 9·0. The resultant solutions retain their original optical rotation. This has been done both for solutions with rotations of —55° and of —52°. Complete preparations were made at pH 6·1 (experiment No. 10 in Table 1), when the edestin obtained was amorphous, and also at pH 8·0 (experiment No. 12 in Table 1). There was no change in specific rotation from the usual results obtained. Extractions carried out at temperatures as high as 60°C. (experiment No. 27) yielded products with higher rotations than those prepared by extraction at 40°C. This last temperature has no apparent effect on denaturation. Preparations made without the application of heat had in some cases a higher specific rotation than those done at 40°C. This is apparently caused by the more prolonged time needed for extraction and crystallization.

Weyl (1877) observed the effect of water on globulin solutions. On long contact with water they became gradually insoluble in neutral salts. Osborne (1902) showed that contact with water produced some insoluble material from edestin. Experiments Nos. 3, 6, and 17-20 in Table 1 show the increase in optical rotation following treatment of edestin with water. After precipitating the protein by the addition of water and allowing it to stand under water, the specific rotation increases to —56° to —58°. Even allowing the edestin crystals to stand

TABLE 1.

Number of experiment.	Temperature of extraction.	Solvent used for extraction.	Solvent used for final solution.	Remarks on the method of preparation.	pH of solution.	Concentration of protein, %	Optical Rotation.	Length of tube, dm.	Specific Rotation.
1	17°C.	10% NaCl	10% NaCl	No heat applied for crystallization	6·8	2·82	1·43	1	−50·7
2	40°C.	10% NaCl	10% NaCl	No preliminary extraction with an organic solvent	6·3	1·65	0·85	1	−51·5
3	—	—	10% NaCl	2 precipitated by H_2O at 15°C. for 24 hours	6·4	1·20	1·38	2	−57·6
4	40°C.	10% NaCl	10% NaCl	Complete preparation in 7 hours	—	1·49	1·58	2	−52·9
5	40°C.	10% NaCl	10% NaCl	Complete preparation in 2 days	6·7	4·01	2·11	1	−52·6
6	—	—	10% NaCl	5 precipitated by H_2O at 15°C. for 2 days	7·3	1·22	1·34	2	−55·0
7	40°C.	10% NaCl	10% NaCl	Complete preparation in 3 days	6·5	3·09	3·10	2	−50·2
8	40°C.	10% NaCl	10% NaCl	Preliminary extraction with petroleum ether	—	2·36	1·20	1	−50·9
9	30°C.	5% $(NH_4)_2SO_4$	5% $(NH_4)_2SO_4$	—	—	2·36	2·44	2	−51·7
10	40°C.	10% NaCl	10% NaCl	—	6·1	1·06	1·11	2	−51·9
11	40°C.	10% NaCl	10% NaCl	—	7·0	2·48	2·60	2	−52·4
12	40°C.	10% NaCl	10% NaCl	—	8·0	1·60	1·65	2	−51·3
13	40°C.	10% NaCl	5% NaCl	—	6·5	0·637	0·64	2	−50·2
14	40°C.	10% NaCl	10% NaCl	—	—	4·48	2·36	1	−52·7
15	40°C.	10% NaCl	15% NaCl	—	—	4·19	2·09	1	−49·9
16	40°C.	10% NaCl	10% NaCl	Complete preparation in 7 hours	6·6	2·20	1·14	1	−51·8
17	—	—	10% NaCl	16 precipitated by H_2O at 0°C. for 2 days	6·6	1·54	1·76	2	−57·2
18	—	—	10% NaCl	17 precipitated by H_2O at 0°C. for 2 days	6·85	2·66	1·52	1	−57·1
19	—	—	10% NaCl	18 precipitated by H_2O at 0°C. for 2 days	—	2·20	1·23	1	−55·9
20	—	—	10% NaCl	19 precipitated by H_2O at 0°C. for 2 days, at pH 9·0	—	1·10	1·26	2	−57·3
21	40°C.	10% NaCl	10% NaCl	Allowed to stand in the refrigerator under dilute NaCl solution between crystallizations	—	1·90	1·04	1	−54·7
22	40°C.	10% NaCl	10% NaCl	" " " " "	6·8	3·58	1·96	1	−54·6
23	17°C.	10% NaCl	10% NaCl	Crystals obtained by dialysis of salt solution	—	2·39	1·32	1	−55·2
24	40°C.	10% NaCl	10% NaCl	Crystals obtained by dialysis from filtrate after one crystallization	6·3	1·49	0·82	1	−55·0
25	40°C.	10% NaCl	10% NaCl	Dried with alcohol and ether	—	6·13	3·12	1	−50·9
26	60°C.	10% NaCl	10% NaCl	Extracted for 30 minutes	6·5	1·55	1·70	2	−54·8
27	60°C.	5% NaCl	Water.	Insoluble in NaCl solution. Is the dihyrochloride form	5·7	2·00	1·18	1	−59·0
28	40°C.	10% NaCl	10% NaCl	Precipitation by alcohol from salt solution at 15°C. dried	6·8	2·10	1·20	1	−57·1
29	40°C.	10% NaCl	10% NaCl	Precipitation by alcohol from salt solution at −5°C. dried	6·7	1·36	1·21	2	−56·4

for 2–3 days suspended in very dilute salt solution at 0°C. (experiments Nos. 21 and 22), produces a protein with a rotation greater than the ordinary value. Crystallization by dialysis after extraction in 10 p.c. salt solution (experiments Nos. 23 and 24), yields a similar product through continued contact with water.

Edestin is not markedly denatured by treatment with organic solvents as in the case of the serum proteins. Protein preparations from hempseed without the preliminary extraction with ether or petroleum ether (experiment No. 2) do not have a lower specific rotation than the ordinary preparations, even when the oil extraction is done at room temperature. Drying the crystals with alcohol and ether also has no apparent effect (experiment No. 25). Precipitation of edestin from a salt solution with absolute alcohol either at 0°C. or at laboratory temperature increases the rotation of the resultant solution in sodium chloride to —56° to —58°, as shown by experiments Nos. 28 and 29.

Treatment with acid, alkali, or heat produces a large increase in the specific rotation of edestin. Results are given in Table 2.

TABLE 2.

Treatment of edestin.	Optical Rotation. 1 dm.	Concentration protein. %	Specific Rotation.
1. N/100 HCl 100°C. 7 minutes	0·75	1·0	— 75
N/100 HCl 100°C. 1 hour	0·83	1·0	— 83
2. N/10 HCl Laboratory temperature 1 week. Dissolved N/100 NaOH	1·44	1·68	— 85·8
3. N/10 HCl 15–20°C. 1 day. Neutralized and precipitated, washed, and dried. Insoluble in water or acid. Soluble in N/100 NaOH.	0·70	0·83	— 84·3
4. N/100 NaOH 100°C. 7 minutes	0·78	1·0	— 78
N/100 NaOH 100°C. 1 hour	0·78	1·0	— 78
5. N/100 NaOH Laboratory temperature. Immediately	1·00	1·55	— 64·7
N/100 NaOH Laboratory temperature. 15 minutes	1·12	1·55	— 72·4
N/100 NaOH Laboratory temperature. 7 hours	1·25	1·55	— 80·9
N/100 NaOH Laboratory temperature. 14 hours.	1·38	1·55	— 93·2
N/100 NaOH Laboratory temperature. Next day	1·15	1·55	— 72·5
6. 10% NaCl solution 70°C. 4 hours. Insoluble precititate dissolved in dilute NaOH	2·40	2·64	— 90·9
7. 10% NaCl solution pH 6·7 100°C. 4 hours. Dissolved N/10 NaOH	0·72(2 dm.)	0·32	—109

As these results show, the highest rotations were obtained by the action of heat, although the rate of coagulation at 100°C. in the presence of 10 p.c. salt solution at pH 6·7–7·0 is very low. Chittenden and Mendel (1894) found that after prolonged heating on a boiling water bath some unaltered edestin remains. This has been confirmed (experiment No. 7 in Table 2). Four hours on a boiling water bath at pH 6·7 coagulated less than half the edestin. This dissolved in dilute sodium hydroxide solution and had a rotation of —109°. The filtrate was recrystallized and its specific rotation was —57·8. This could be coagulated on more prolonged boiling.

Some edestin crystals were dissolved in dilute acetic acid (pH 5·4) without

the addition of sodium chloride. The specific rotation was —72°. On neutralization of this solution to pH 7·5 the rotation fell to —56·6°. More drastic treatment with acid or alkali produced substances quite insoluble in salt or acid solution, and with higher specific rotations. These rotations were approximately of the same order when either acid or alkali was used. Racemization occurred in the alkaline experiments after the initial increase in rotation.

DISCUSSION.

The fact that specimens of edestin prepared by methods which might involve a small amount of denaturation have a somewhat increased rotation compared with that of a specimen prepared with the greatest care may be susceptible of two explanations. The first is that the molecules of the active protein have undergone a small change in constitution, sufficient to affect their rotation but not their ability to crystallize. The second is that a small proportion of the protein has been denatured and remains associated with the crystals of native edestin. Experiments in this laboratory (1933 and 1934) show that native proteins in the presence of neutral salts can dissolve considerable quantities of metaproteins, even at their isoelectric point.

The second explanation does not conflict with any of the ascertained properties of proteins. If the first be true, it is, as yet, the only known case. A method of discrimination between these hypotheses is not yet available.

SUMMARY.

Specific rotations of crystalline edestin prepared by various methods have been observed. The figure obtained for quickly-prepared edestin is —50° to —53°. Edestin prepared by slower methods, e.g. dialysis, by alcohol precipitation, or by long contact with water has a specific rotation of —56° to —58°.

This is probably due to a mixture of some metaprotein with the native edestin.

Higher rotations can be observed by treatment of edestin with acid, alkali, or heat.

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"SMOOTH-ROUGH" VARIATION IN *PHYTOMONAS MEDICAGINIS PHASEOLICOLA* BURK

by

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Arkwright (1930) has reviewed the data concerning the "Smooth-Rough" *(S-R)* variation in bacteria. As the phenomena were first noted and most extensively observed in animal pathogens, it is natural to expect that most of the recorded observations on variation should refer to these organisms. This is true particularly of the Gram-negative group of intestinal organisms.

Actually, apparent cases of the *S-R* variation have been observed in a number of plant pathogens. Sharp (1927) describes the isolation of smooth and rough strains of *B. phaseoli sojense* from a pure culture which macroscopically had appeared to be smooth. He states that the two strains were identical physiologically and serologically, but differed morphologically, in virulence and in range of acid agglutination. Each strain remained constant in culture media, but both types were present in re-isolation from plants inoculated with *R*. For the soya bean the *R* strain was less virulent when measured by the time of appearance and the size of the lesions. However, it is questionable whether Sharp was working with a typical *R* form, because in addition to his failure to demonstrate a serological difference between the *R* and *S* types of *B. phaseoli sojense* his photographs of the *R* type are rather different from those of the *R* type of the Gram-negative group of intestinal organisms.

Link and Hull (1927) mention that roughness was observed for *B. citri*, *B. medicaginis phaseolicola*, *B. phaseoli sojense*, and *B. tumefaciens*. Bryan (1928) refers to a rough form of *B. syringae* which, compared with the normal form, was very weakly pathogenic. Kauffmann (1928) obtained a rough form of *B. tumefaciens* differing in serological characters from the smooth form. He also found that both his rough and smooth forms were susceptible to a bacteriophage obtained from faecal extracts. Muncie and Patel (1930) report a rough form obtained from an ordinary culture of *B. tumefaciens* to which a bacteriophage had been added.

Elcock (1931) in a study of *P. beticola* found a strain exhibiting dissociation

to *R* and *S* forms. Both forms were pathogenic to sugar beets, but *R* produced the larger overgrowths. His forms were very unstable. Rough and smooth strains of *B. tumefaciens* and *B. phytophthorus* were obtained by Quirk (1931), while Israilsky and Starygin (1930) report roughness for *B tumefaciens* and for *B. radicicola.*

The organism upon which our observations have been made is the cause of the Halo Blight disease of French Beans *(Phaseolus vulgaris).* This disease was first distinctly recognized by Burkholder (1926), who proved that the etiologic agent was a bacterium which he named *Phytomonas medicaginis var. phaseolicola,* hereinafter referred to as *P.m.p.*

P.m.p. may invade all parts of the bean plant, including the seed. The organism lodges under the seed coat, and in a severe infection produces visible lesions on white seeds, so that, after thorough superficial sterilization of such seeds, pure culture isolations of the organism can be readily secured. Isolated from such a source, colonies of *P.m.p.* on beef extract agar appear within 48 hours if incubated at 27°C. Typical colonies, at first, are white, smooth, round, and glistening with an entire margin. When obliquely illuminated they show internal cross hatchings. Later, they may become concentrically ringed and finely granular with a crenate margin.

In the course of making isolations from a batch of infected "Magnum Bonum" seeds it was noticed, seven days after the first appearance of typical colonies of *P.m.p.* on beef extract agar, that some colonies had produced secondary or daughter colonies as papillae on the parent or mother colonies. These daughter colonies were sometimes scattered irregularly over the surface, and sometimes confined to the circumference. Sub-cultures from such colonies yielded two distinct types of colonies; "roughs" and "smooths".

We have since found rough colonies appearing as secondary growths after lysis of the smooth strain by a bacteriophage isolated from bean seeds infected with *P.m.p.* We have tried to secure the *R* form by subculturing from old broth cultures of the *S* form (up to 9 months old), and by growing the *S* form in its homologous antiserum, but neither method has yielded the *R* form.

VARIATIONS CORRELATED WITH THE *S-R* PHASE CHANGE.

1. *Appearance of Colonies on agar.*

The *S* form, which may be regarded as normal, has already been described. A typical *R* form of colony is usually a little larger than the *S* form, has a wavy margin and a crinkled dry-looking surface.

2. *Agglutination of a Non-Specific Character.*

(1) *Growth in Broth.*—Incubation of a nutrient broth subculture of bacteria from an *S* colony results in a uniformly turbid growth indicating an even dispersal of bacteria in the fluid. In contrast with this, a subculture of bacteria from an *R* colony grows in clumps which tend to settle and leave the culture fluid clear. Microscopic examination of both forms as grown in broth shows them to be motile.

(2) *Agglutination in Saline Solutions.*—One of the criteria that distinguishes the *R* and *S* forms in animal bacteriology is the relative sensitivity of suspensions

to different concentrations of sodium chloride. Figures relating to an experiment to test the sensitivity of the two phases of *P.m.p.* to different salt concentrations are given in Table 1 below. For this experiment heavy suspensions of *P.m.p.-R* and *P.m.p.-S* were made up in distilled water, and single drops were added to tubes containing NaCl solutions of the concentrations indicated. Readings were taken on the following morning after the suspensions had stood overnight at room temperature.

TABLE 1.

Agglutination of R and S Forms at Different NaCl Concentrations

Phase.	Salt concentrations, NaCl p.c.								
	0·0	0·2	0·85	1·0	1·5	2·0	5·0	10·0	20·0
S	–	–	–	–	–	–	–	–	–
R	–	–	–	+	+	+	+	+	+

+ = agglutination.

Thus at all salt concentrations the *S* form produced stable suspensions which did not "salt out," even at very high concentrations.

The *R* form produced a stable suspension in normal saline. In this it differs from most described *R* variants, being more stable than the average. However, with slightly greater concentrations of saline the *P.m.p. -R* agglutinated. This agglutination by salt appeared to be associated with the body of the bacterium rather than with the flagella, for when examined microscopically the clumps could be seen being carried about by the flagella which were free and active.

The stability of the *R* form in high concentrations of NaCl could be secured by extracting the bacteria with absolute alcohol. On the other hand by heating the *R* suspension at 100° C. for 1 hour its sensitivity to saline was increased and it became unstable in 0·2 p.c. saline, though stable in distilled water. Heating, however, did not affect the stability of *S* suspensions. Extraction with alcohol will not render the *R* strain stable if, prior to this treatment, it has already been heated at 100°C.

(3) *Agglutination in Bean "Sap."*—Fresh bean pods were crushed and the extract filtered through a Seitz EK filter. The clear filtrate had a pH of 6·7 and an ash content of 0·5 p.c.

The stability of the *R* and *S* forms was tested in the filtrate with the following results:

TABLE 2.

Stability of R and S Forms in Bean "Sap".

Phase.	(a) Undiluted "sap".	(b) "Sap" diluted ½.	(c) After treating the undiluted "sap" at 100°C. for 15 min.
S	–	–	–
R	+	–	+

+ = agglutination.

The agglutination of *P.m.p. -R* in "bean sap" is apparently an expression of the sensitiveness of this form to the electrolytes contained in the sap.

(4) *Agglutination in 0·2 p.c. trypaflavine.*—Pampana (1933), working with several species of bacteria, observed that the "roughness" or "smoothness" of single colonies could be determined by noting whether or not agglutination occurred in a 0·2 p.c. solution of trypaflavine in normal saline. These results were confirmed for the *R* and *S* forms of *P.m.p.*

3. *Agglutination of a Specific Character.*

It is generally agreed that the change from the *S* to *R* form involves an alteration in the structure of the somatic antigen, and that the demonstration of such an alteration by serological means is the surest evidence of the *S-R* phase change.

In our experiments on specific agglutination antisera were prepared, the details of which are summarized below.

P.m.p. -S and *P.m.p. -R* were grown on a solid medium consisting of Fermi's solution to which 2·0 p.c. agar was added. Suspensions for the inoculations were prepared by washing off with normal saline (10 c.c.) the growth from cultures, two days old, grown on slopes in test tubes.

Two rabbits were inoculated with the suspensions of the living bacteria as follows:

Date.	Rabbit A.	Rabbit B.
17/7/33	3 c.c. intraperitoneally	3 c.c. intraperitoneally
19/7/33	2 c.c. intravenously	2 c.c. intravenously
22/7/33	2·5 c.c. intravenously	2·5 c.c. intravenously
25/7/33	3·5 c.c. intravenously	3·5 c.c. intravenously

On 27/7/33 trial bleedings were taken from the ears, and on 28/7/33 the rabbits were killed and blood obtained from the heart. After allowing it to clot in the incubator at 37°C. for 1 hour, the sample was placed in the ice-chamber overnight. Serum was collected next day, centrifuged, and, without glycerination, stored ready for use.

Agglutination Tests with Untreated S and R Suspensions.—For these tests the *S* form was suspended in 0·85 p.c. and the *R* form in 0·2 p.c. saline. The tubes were incubated at 37°C. and readings taken after 4 and 24 hours. The results are shown in Table 3. Agglutination in all cases was of a flocculent nature. Normal rabbit's serum did not agglutinate either form.

TABLE 3.

Agglutination of Untreated S and R Suspensions.

Results using S antiserum.

Suspension.	Final dilution of serum.										
	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960
P.m.p. -S	+++	+++	+++	+++	+++	+++	+++	+++	+– –	– – –	– – –
P.m.p. -R	+++	+++	+++	+++	+++	+++	+++	+– –	– – –	– – –	– – –

TABLE 3 (continued).

Agglutination of Untreated S and R Suspensions.

Results using R antiserum.

Suspension.	Final dilution of serum.										
	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960
P.m.p. -S	+++	+++	+++	+++	+++	+++	+++	+++	+++	++−	−−−
P.m.p. -R	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++−

Controls: *S* serum plus saline −−− *R* serum plus saline −−−
S suspension plus saline −−− *R* suspension plus saline −−−

These tables show that each form reacted to a high titre with each of the two antisera and that the organisms cannot be differentiated by these tests. The probable reason for this similarity in behaviour is the fact that both the forms used to produce the antisera were motile. Since flagella have antigenic qualities it is to be expected that antibodies specific for this antigen would be present in both sera and that reactions in which the flagellar antigen *(H)* took part would tend to mask reactions depending on other differences of antigenic structure. Subsequent experiments suggest that this is the correct explanation.

Agglutination Tests with Heated S and R Suspensions.—To differentiate between the two forms it is necessary to eliminate the flagellar antigen *(H)* before comparing the somatic antigens. White's method of treating the suspensions with alcohol to destroy the flagellar antigen rendered both strains of *P.m.p.* largely inagglutinable and could not be used. Attempts to secure non-motile strains by growth on phenolized agar were also unsuccessful. However, the flagellar antigen is heat-labile, so that agglutination tests were carried out with bacterial suspensions which had previously been heated at 100° C. for 1 hour. As heated suspensions of *P.m.p. -R* agglutinate spontaneously in 0·2 p.c. saline, the tests with this form were carried out with suspensions in distilled water. The *S* form was suspended in 0·85 p.c. saline. The results are tabulated in Table 4.

TABLE 4.

Agglutination of Heated S and R Suspensions.

Results using S antiserum.

Suspension.	Final dilution of serum.									
	40	80	160	320	640	1,280	2 560	5,120	10,240	20,480
Heated *P.m.p. -S*	+++	+++	+++	+++	+++	+++	+++	+++	++−	−−−
Heated *P.m.p. -R*	+++	+−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−

The agglutination was of a fine sedimentary nature.

Results using R antiserum.

Suspension.	Final dilution of serum.									
	40	80	160	320	640	1,280	2 560	5,120	10,240	20,480
Heated *P.m.p. -S*	+++	+++	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−
Heated *P.m.p. -R*	+++	++−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−

Controls: *S* serum plus saline −−− *R* serum plus saline −−−
S suspension plus saline −−− *R* suspension plus saline −−−

From this experiment, which has been repeated with similar results, it is clear that *P.m.p. -S* and *P.m.p. -R* can be differentiated on the basis of their comparative reactions with the *S* antiserum, indicating the presence of an antigenic substance in the heated suspension of *P.m.p. -S* which is not present in the heated suspension of *P.m.p. -R*. This difference is further confirmed by the fact that untreated *P.m.p. -R* suspensions used as the antigen to prepare the *R* antiserum failed to produce agglutinins which would react to a high titre with the heated *P.m.p. -S* suspension.

The failure of the heated suspensions of *P.m.p. -R* to react to a high titre with its homologous antiserum may be due to the failure of *P.m.p. -R* to provoke somatic agglutinins, or to the fact that the *R* somatic antigen is in some degree heat labile, or else to the lack of electrolytes in the suspensions used for the test.

4. *Sensitivity to a Bacteriophage.*

By suitable modifications of d'Herelle's (1926) technique, a bacteriophage was isolated from infected "Magnum Bonum" bean seeds. From the lytic filtrate a pure strain of 'phage was cultivated for use in further experiments. This was a pure filtrate in that it was developed from a single plaque. So far as tested, this 'phage has proved specific for *P.m.p.*, including a strain isolated in England.

The lytic action of the 'phage was tested in the following manner. Agar plates were flooded with a saline suspension of the organism under examination. After thoroughly draining the excess of the suspension from the plates, spots on the agar surface were touched with a platinum needle which had been dipped in the 'phage filtrate.

Tested in this way films of *P.m.p. -S* showed well defined lysis at the points of inoculation which was indicated by bare areas surrounded by the bacterial film of growth, while no such reaction occurred with films of *P.m.p. -R*. In the lysed areas of the *P.m.p. -S* plates, there appeared later, "rough" secondary colonies which proved to be 'phage resistant. The difference in sensitiveness of the *R* and *S* forms to this bacteriophage conforms to the hypothesis of Burnet (1930), that the entity which functions in serological reactions as the somatic antigen determines, in some way, whether lysis will take place, possibly by a specific adsorption of the 'phage.

In the lysed areas of *P.m.p. -S* the vast majority of the secondary colonies were "roughs." On a few plates, however, there appeared a few small colonies of a variant distinct from both *P.m.p. -S* and *P.m.p. -R*. These colonies grew very slowly on agar, were small, round, smooth, of a coherent consistency, and often failed to grow on subculturing to fresh media. In nutrient broth growth was scanty and flocculent with a slimy deposit. Suspensions were stable in 0·85 p.c. saline but agglutinated after heating at 100°C. Extensive inoculation experiments with this form failed to produce lesions on either the stems, leaves, or pods of the bean plant.

The growth characteristics and behaviour of this variant recall those of a rough variant of *B. dysenteriae* mentioned by Arkwright (1921), who states: "One other type of colony *'RV'* must be described, however, since it was definitely associated with the property of forming clear cultures with a deposit in broth and of agglutinating spontaneously in salt solution. These colonies were very small, round and smooth, and of an unusually coherent or sticky consistency."

5. *Virulence.*

Virulence can be taken to denote the capacity of a pathogenic micro-organism to cause the death, or to induce a pathological condition in a part or the whole, of the host plant.

Variation in the virulence of bacteria is now well recognized for animal pathogens, and it has been shown that such differences may be correlated with colony form. Thus the *R* form of *B typhosus* is much less virulent than the *S* form, a type of relationship which holds for many other organisms.

There is no simple and accurate method for comparing the virulence of bacterial plant pathogens. There are difficulties associated with the standardization of the bacterial dosage actually given, and the manner in which the organism is introduced into the host plant. In comparing the virulence of the *R* and *S* forms of *P.m.p.* we have not attempted to meet all these difficulties.

Experiments with Stem Inoculations.

In the first experiment separate individual plants were inoculated by needle punctures at the first leaf node with inoculum from young agar cultures of the *S* and *R* forms of *P.m.p.* Two to three weeks later symptoms of disease appeared on all of the inoculated plants. They were stunted, with yellow terminal leaflets (indicating a systemic infection), and frequently there were gummy bacterial exudates on the stems. Ultimately the plants wilted, although in some cases plants inoculated with the *R* form survived to flower and set seed. The progress of the disease was always more rapid, and wilting occurred sooner in plants inoculated with the *S* form than in those inoculated with *R*. Re-isolations from plants inoculated in these ways resulted in the recovery of the original forms used; there was no evidence of a transformation of forms within the host plant.

In a later series of stem inoculation experiments, larger numbers of plants were inoculated under similar conditions using the two different forms. In these cases the severity of the disease varied from plant to plant. Eighteen days after inoculation the plants were placed in a row in the order of the severity of development of wilting symptoms but without regard to the inoculum used. Table 5 below indicates the source of the inoculum, while the numbers show the order in which the plants were arranged, the low numbers indicating complete wilting and the higher numbers a less degree of wilting.

TABLE 5.

Inoculation of Bean Seedlings with S and R Forms.

	Series (a).	Series (b).
S form	(1, 2, both dead), 3, 4, 6, 7.	(1, dead), 2, 3, 4, 5.
R form	5, 8, 9, 10, 11, (12, not wilted).	6, 7, 8, (9, 10, not wilted).

Isolations were made from all inoculated plants and in no case was there evidence of an alteration in the phase of the form used for inoculation.

Pod Inoculation Experiments.

In these experiments a number of pods growing on the same plant were inoculated at several points at random with *S* and *R* forms, while other points on the same pods were pricked with a sterile needle to serve as controls.

An experiment commenced on 13.12.33 may be quoted to show the type of result obtained. On each of four pods, inoculations were made by needle pricks at two points with *S*, at two points with *R*, and at two other points with a sterile needle.

Table 6 shows when the lesions resulting from these inoculations were first noticeable. The controls showed no lesions at the end of the experiment.

TABLE 6.

Inoculation of Bean Pods with S and R Forms.

Time elapsing since inoculation in days.	Number of lesions developed.	Character of inoculum.
5	6	*S*
6	2	*S*
7	2	*R*
8	4	*R*
9	2	*R*

From this table it is clear that the *S* form produces noticeable lesions more rapidly than the *R* form.

Re-isolation showed that in all cases the organism present in the lesions was of the same form as that used for inoculation.

Experiments with Leaf Inoculations.

In these experiments young plants growing in pots were sprayed by means of an atomizer with suspensions of the *S* and *R* forms. The conditions and results of such an experiment are shown below.

The experiment was commenced on 27.6.33. Twelve bean plants were sprayed with a suspension of the *S* form, while another twelve plants were similarly inoculated with the *R* form. They were then placed in a moist chamber maintained at 10°C. for 6 hours before being transferred to the greenhouse bench. After 15 days the plants were examined with the results shown in Table 7.

TABLE 7.

Spray Inoculations of Bean Seedlings with S and R Forms.

Type of inoculum used.	Number of plants		
	Severely infected.	Lightly infected.	Not infected.
S	8	2	2
R	8	4	0

The heavily infected plants were distinguished from those lightly infected by the greater number of stomatal infections and by the fact that the next youngest leaves to those with stomatal infections instead of showing the normal green colour characteristic of healthy leaves were yellow, indicating a more generalized infection.

The experiments designed to throw light on the comparative virulence of the *S* and *R* forms of *P.m.p.* have not brought to light substantial differences in this quality, though there is a small and consistent difference between the two forms indicating a greater virulence of the *S* form.

SUMMARY.

The plant pathogen *Phytomonas medicaginis phaseolicola* Burkholder may exist in at least two forms, *S* and *R*, which differ from one another essentially in the same respects as do the now well-recognized *S* and *R* forms of the Gram-negative intestinal organisms.

Occurring simultaneously with this *S-R* change in colony form are variations in non-specific and specific agglutination, in sensitivity to a bacteriophage, and in virulence.

The *R* form gives a flocculent growth in broth, is relatively unstable in salt solutions (much more so after heating at 100°C.) and in undiluted bean "sap"; it is also agglutinated in 0·2 p.c. trypaflavine. The *S* form gives a uniform turbidity in broth, is stable in salt solutions both before and after heating, and also in bean "sap"; it is not agglutinated in 0·2 p.c. trypaflavine.

It has been demonstrated by means of the agglutination test that the change from the *S* to *R* form involves a change in the heat stable or somatic antigen.

The *S* strain was sensitive and the *R* strain non-sensitive to a bacteriophage obtained from diseased bean seeds.

The *R* strain was less virulent than the *S* strain, while a third variant designated "*RV*" was proved to be non-pathogenic to the bean plant.

ACKNOWLEDGMENTS.

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THE ABSORPTION SPECTRA OF HYDROXY-NAPHTHAQUINONES AND OF THE COLOURING MATTER OF *DROSERA WHITTAKERI*

by

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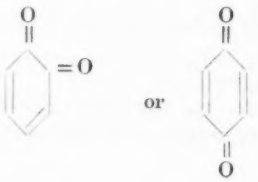
The investigation of the colouring matters occurring in the insectivorous plant *Drosera Whittakeri*, was first undertaken by Rennie (1887, 1893), who published two papers on the subject but never completed the work.

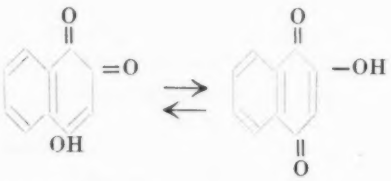
The crude colouring matter can be isolated from the dried bulbs by repeated treatment with boiling alcohol until the extract is only pale red in colour. The greater part of the solvent is then distilled off and the colouring matter, together with a fat-like substance, precipitated by the addition of water. Several sublimations of the material so obtained are necessary before all traces of the fatty substance are entirely eliminated. By careful fractional crystallization of the colouring matter it is possible to separate it into two closely-related components, one of which is much less soluble in glacial acetic acid or alcohol than the other. The later stages of the separation are greatly facilitated by acetylation, as the difference in the solubilities of the acetates is more marked. The free dye-stuff can be regenerated from the acetate by saponification with caustic soda solution followed by the addition of dilute hydrochloric acid.

From a study of the reactions and molecular weights of these two substances, Rennie concluded that the less soluble was a trihydroxy-methyl-naphthaquinone and the other a dihydroxy-methyl-naphthaquinone. It is now proposed that these be named hydroxy droserone and droserone respectively.

Unfortunately the chemical behaviour of the compounds does not supply any conclusive evidence as to which type of quinone structure is present, and most of the criteria proposed in the past are far from satisfactory.



Caspari (1932) discussed the crystallography of some simple quinones, but his survey of the data and structure appears to reveal no regularities or general characteristics for quinones as a class. The colour of a quinone or quinone derivative has been employed to deduce the ortho (1:2) or para (1:4) structure of the nucleus, the former type being more highly coloured than the latter. Hooker (1896), Fieser and Ames (1927); see also Pummer and Prell (1922), Pummer and Fiedler (1927). This test, however, is unreliable as some diaryl p-benzoquinones have been obtained in both red and yellow modifications. Also the introduction of hydroxyl groups into the nucleus modifies the colour so greatly that no reliance can be placed on such a factor as a guide to quinonoid constitution, e.g. naphthazarin (5:8-dihydroxy-1:4-naphthaquinone), whose structure is unequivocal, is decidedly red in colour. Again the presence of hydroxyl groups further complicates the question on account of the undoubted tautomerism which exists. Fieser (1926, 1928) has shown that 4-methoxy-1:2-naphthaquinone is quickly and completely converted into 2-methoxy-1:4-naphthaquinone by warming with methyl alcohol containing a small percentage of hydrochloric acid; and by a study of reduction potentials he has deduced that in the equilibrium



only 0·2 p.c. of the 1:2-quinone is present in an aqueous alcoholic (37 p.c. alcohol) solution. In general he concludes that in the tautomeric forms of hydroxy quinones in solution, that with the lowest reduction potential will predominate. To all intents this is experimental support of the statement that in general para quinones are more stable than ortho (Dimroth and Kerkovius, 1913).

The object of the present work is to investigate the possibility of distinguishing between the o- and p-quinones by spectroscopic methods and, by use of the results so obtained, to gain some information as to the constitution of hydroxy droserone. Unfortunately the purity of the specimens of droserone so far prepared is doubtful, so that its absorption spectrum has not been recorded.

Methods of Preparation of the Substances Examined.

Hydroxy Droserone. This was isolated as previously described. It separates from glacial acetic acid as red plates. M.P. 192°C.

Hydroxy Droserone Triacetate. The colouring matter was boiled for a short time with acetic anhydride and a fragment of anhydrous zinc chloride, until the colour changed from red

to yellow. On cooling the triacetate separates, and may be recrystallized from glacial acetic acid. The substance so obtained contains solvent of crystallization, which must be driven off by heating at 120°C. for half an hour. M.P. 152-153°C.

2-Methyl-1: 4-Naphthaquinone. Madinaveitia and de Buruaga (1929). 2-Methyl-naphthalene in acetic acid solution, was oxidized in the cold with chromic acid. Yellow needles, M.P. 106°C.

2-Hydroxy-1: 4-Naphthaquinone. Fieser (1926). 1: 2-Naphthaquinone-4-sulphonic acid was hydrolyzed with sulphuric acid and methyl alcohol, and the 2-methoxy-1: 4-naphthaquinone so formed warmed with 2·5 N. caustic soda. On acidification, the hydroxy quinone was thrown down as a yellow precipitate. Yellow needles from alcohol containing a trace of acetic acid. M.P. 192°C.

2-Acetoxy-1: 4-Naphthaquinone. 2-Hydroxy-1: 4-naphthaquinone was heated with acetic anhydride containing a little anhydrous zinc chloride. Crystallized from alcohol. M.P. 131°C.

2-Methoxy-1: 4-Naphthaquinone. Fieser (1926). On refluxing 2-hydroxy-1: 4-naphthaquinone with methyl alcohol containing 3 p.c. of hydrochloric acid gas, it was rapidly converted into the methyl ether. Recrystallized from water. M.P. 183°C.

4-Methoxy-1: 2-Naphthaquinone. Fieser (1926). On alkylation of the silver salt of 2-hydroxy-1: 4-naphthaquinone with methyl iodide, a mixture of 2-methoxy-1: 4-naphthaquinone and 4-methoxy-1: 2-naphthaquinone was obtained. These were separated by extraction with sodium bisulphite solution in which the 4-methoxy compound is soluble. Addition of sodium carbonate to this solution precipitated the 1: 2-quinone ether. Orange-red needles from benzene. M.P. 190°C.

Juglone. Willstätter and Wheeler (1914). A paste of 1: 5-dihydroxy-naphthalene was added to dilute chromic acid, the temperature being kept below 10°C. Next morning the gelatinous precipitate was filtered off and dried *in vacuo*. It was then extracted with petroleum ether from which the juglone crystallized as yellow needles. M.P. 150-150·5°C.

Juglone Acetate. Bernthsen and Semper (1885). Juglone was refluxed for eight hours with acetic anhydride, and the mixture then poured into water. Pale yellow needles from alcohol. M.P. 154°C.

Hydroxy Juglone. Willstätter and Wheeler (1914). Juglone, suspended in ether, was reduced with zinc dust and 2 N. sulphuric acid until the yellow colour disappeared. The ethereal solution was then oxidized with alkaline ferricyanide, the crude hydroxy juglone being precipitated by the addition of acid. The substance was purified by extraction with benzene, and crystallization of the sodium salt. Yellow plates from glacial acetic acid, melting at 220°C., with charring and evolution of gas.

Naphthazarin. This was an I.G. product. Purification was effected by repeated crystallization from glacial acetic acid or from alcohol.

Naphthazarin Diacetate. Wheeler and Edwards (1917). This substance was prepared by prolonged refluxing of naphthazarin with acetyl chloride. Yellow prisms from glacial acetic acid. M.P. 190°C.

Isonaphthazarin. Zincke and Ossenbeck (1899). 1: 2-Naphthaquinone was oxidized with a solution of bleaching powder containing about 4 p.c. of hypochlorous acid. The solution was then made just acid with acetic acid, filtered, treated with a few c.c. of hydrochloric acid, and warmed, when the isonaphthazarin was precipitated. Orange red plates from glacial acetic. M.P. 280°C.

Isonaphthazarin Diacetate. Zincke and Ossenbeck (1899). Isonaphthazarin was refluxed for a short time with acetic anhydride. White needles. M.P. 105°C.

Carminazarin (8-Methyl-2: 3: 6-trihydroxy-1: 4-naphthaquinone-5-carboxylic acid). Dim-

roth (1919). Carminic acid was treated at 0°C. with a solution of potassium permanganate acidified with sulphuric acid. The mixture was then heated on a water-bath, when carbon dioxide was evolved, and after cooling was extracted with ether. When crystallized from water, red needles, melting with decomposition at 240-250°C., were obtained.

The absorption spectrum of 1:2-naphthaquinone has been examined by Goldschmidt and Graef (1928), and that of 4-methoxy-1:2-naphthaquinone is shown in Fig. 2. The curve for 1:4-naphthaqinone, Purvis (1912), has been extended and is recorded in Fig. 1; also the absorption curve of 2-methyl-1:4-naphthaquinone is shown in Fig. 2. This has been previously examined by Gomez (1931), but was not available. These may be regarded as typical spectra for the two classes of quinones.

As many of the hydroxy-naphthaquinones were found to decompose under the action of ultraviolet light, it was thought advisable to carry out a preliminary qualitative examination of the naphthaquinones and their derivatives, and it is these results which are considered in the following pages.

The absorption curves of the hydroxy-naphthaquinones differ widely from those of the parent type, probably on account of rearrangements due to tautomeric hydrogen atoms. In many cases there is a decrease in the persistence of the characteristic quinone band and the development of a new band with its head lying between $1/\lambda$ 3520 and 3820. The absorption of naphthalene shows a series of bands between $1/\lambda$ 3500 and 3900; and it seems possible that the bands mentioned above represent a vibration characteristic of the parent hydrocarbon. Stewart and Baly (1906) examined the absorption spectra of a number of substituted benzo-quinones and found that the substitution was accompanied by a decrease of persistency of the characteristic quinonoid band and the development of a new band supposedly characteristic of the benzenoid structure. In this change they saw an explanation of the decrease in reactivity of the carbonyl groups on substitution of the quinone nucleus which Kehrman (1888, 1889), had experienced when examining oxime formation.

EXPLANATION OF FIGURES.

Ordinate: Log thickness (in mm.) of M/100,000 solution.
Abscissa: Wave numbers per mm.

Fig. 1.

——— 1:4. Naphthaquinone.
— — — 2 Hydroxy-1:4-Naphthaquinone in 10% EtOH.
. . . . . Naphthazarin (5:8-Dihydroxy-1:4-Naphthaquinone) in 10% EtOH + NaOEt.

Fig. 2.

——— 2 Methyl-1:4-Naphthaquinone.
— — — 4 Methoxy-1: 2-Naphthaquinone.
. . . . . 7 Hydroxy-1:2-Naphthaquinone.

Fig. 3.

——— 2-Hydroxy-1:4-Naphthaquinone.
—·—·— 2 Acetoxy-1:4-Naphthaquinone.
. . . . . Sodium salt of 2 Hydroxy-1:4-Naphthaquinone in water.

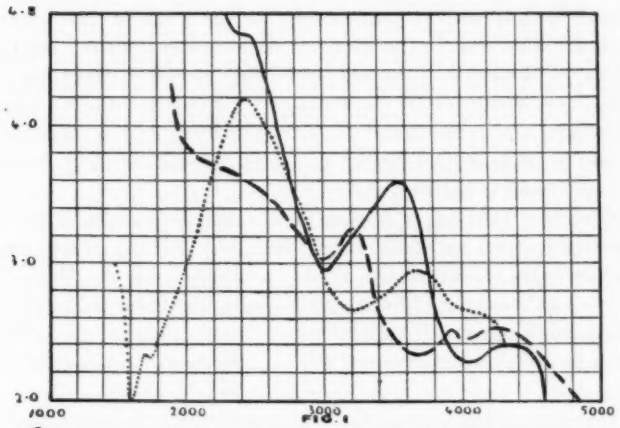


FIG. 1

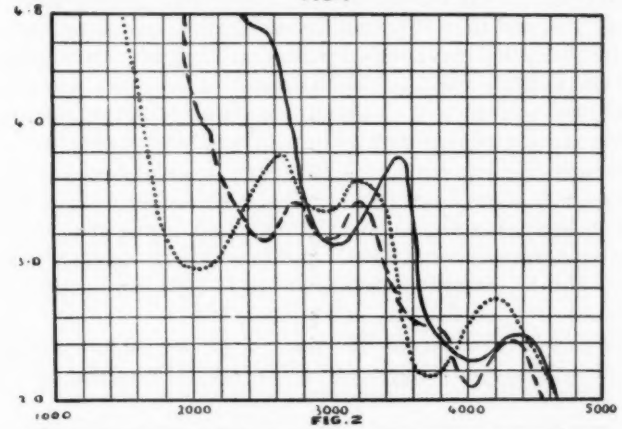


FIG. 2

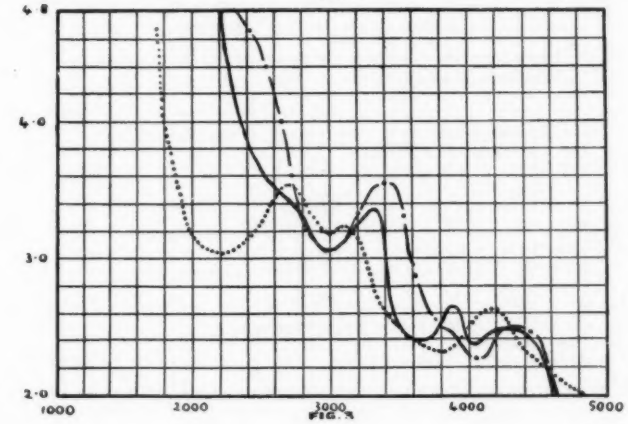


FIG. 3

The band development is most marked in our work in those cases where the hydroxyl group is present in the quinone nucleus, e.g. hydroxy juglone (2 : 5- or 3 : 5-dihydroxy-1 : 4-naphthaquinone) and 2-methoxy-1 : 4-naphthaquinone, Figs. 5 and 4; but its substitution in the benzenoid nucleus gives rise to bands of only slight persistence as in naphthazarin (5 : 8-dihydroxy-1 : 4-naphthaquinone) Fig. 5. In the case of juglone (5-hydroxy-1 : 4-naphthaquinone) Fig. 6, with only one substituent, the band is entirely absent.

At first sight it might appear that these examples furnished further support for the results recorded by Stewart and Baly, but that the effect is not merely a question of an extra substituent is readily apparent, for the absorption spectra of the acetates of these hydroxy-naphthaquinones all revert to the parent quinone type; see isonaphthazarin (2 : 3-dihydroxy-1 : 4-naphthaquinone) and its acetate, Fig. 4; naphthazarin and its acetate, Fig. 5; 2-hydroxy-1 : 4-naphthaquinone and acetate, Fig. 3.

A comparison of the curves for 2-hydroxy-1 : 4-naphthaquinone in alcohol, in 10 p.c. alcohol, and the sodium salt in water gives some information as to the origin of the absorption occurring in the region $1/\lambda$ 2200. The sodium salt shows a definite band at 2200, Fig. 3, which is present in the 10 p.c. alcohol solution of the free hydroxy quinone only as an inflexion, Fig. 1; but in absolute alcohol is absent unless the inflexion at 2620 is in some way related, Fig. 3. A somewhat similar comparison in the region $1/\lambda$ 1630 may be made between the absorption of naphthazarin in alcohol and in aqueous alcohol containing sodium hydroxide. Naphthazarin itself shows an inflexion at 1630, but in the presence of alkali exhibtis a very persistent band at 1630, together with one slightly less persistent at 1750, Figs. 5 and 1. Evidently the absorption in these cases is in some way due to ionization.

For purposes of comparison the position of the various absorption maxima of the naphthaquinones and their derivatives are recorded in the following table:

EXPLANATION OF FIGURES.

Ordinate: Log thickness (in mm.) of M/100,000 solution.
Abscissa: Wave numbers per mm.

Fig. 4.

———— *Iso* Naphthazarin (2: 3-Dihydroxy-1: 4-Naphthaquinone).
— — — *Iso* Naphthazarin diacetate.
· · · · · · 2 Methoxy-1: 4-Naphthaquinone.

Fig. 5.

———— Naphthazarin (5: 8-Dihydroxy-1: 4-Naphthaquinone).
— — — Naphthazarin diacetate.
· · · · · · Hydroxy (3: 5-Dihydroxy-1: 4-Naphthaquinone).

Fig. 6.

———— Juglone (5-Hydroxy-1: 4-Naphthaquinone).
— — — Juglone acetate.
· · · · · · Carminazarin.

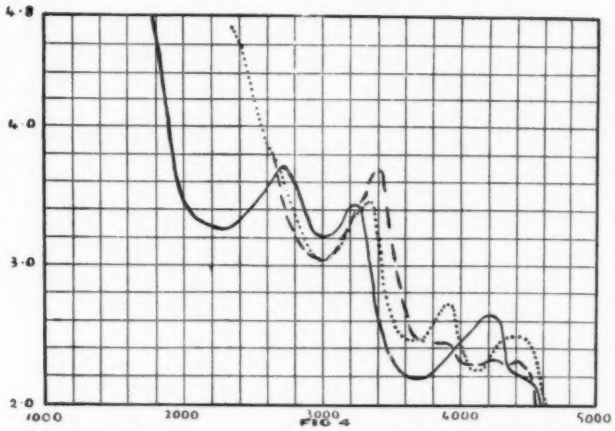


FIG. 4

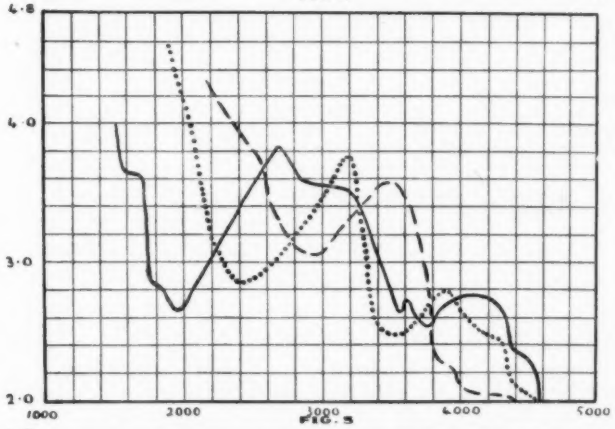


FIG. 5

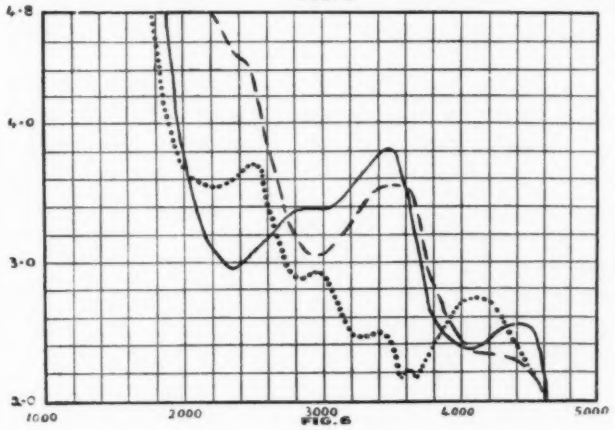


FIG. 6

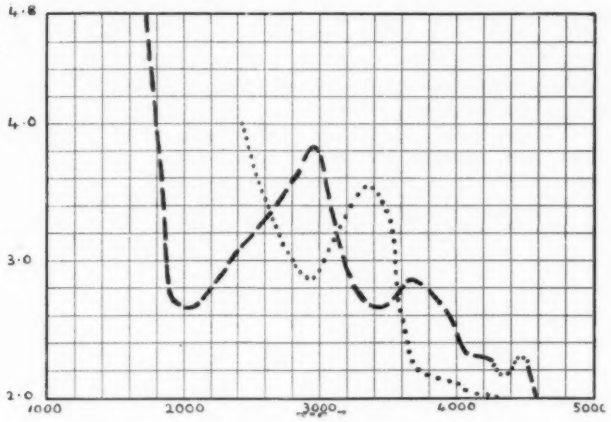
1: 2-Naphthaquinone	2500	2980			4000	
4-MeO-1: 2-N.Q.	2480	2980		(3680)	4000	
7-OH-1: 2-N.Q.	c. 2020	2960		3700		
1: 4-Naphthaquinone	(2420)	2990			4080	
2-Me-1: 4-N.Q.	c. (2460)	3020			4040	
2-AcO-1: 4-N.Q.	(2440)	2980			(3850)	
					4070	
Juglone acetate	c. (2360)	2960			4120	
Naphthazarin diacetate		2950			(3900)	
					(4120)	
Isonaphthazarin diacetate		2960		(3800)	4080	c. 4350 (?)
Juglone	2370	c. (2910)			4060	
Hydroxy juglone	2420			3520	(4120)	(4420) (?)
2-Hydroxy-1: 4-N.Q.	c. (2620)	2990		3650	4020	
Naphthazarin	(1630)	(3030)		3570		(4420)
	(1830)			3750		
	1950					
Isonaphthazarin	2270	3000		3700		(4390)
Carminazarin	2200		3290	3580		
	2850			3690		
2-OH-1: 4-N.Q. in 10% alcohol	c. 2300	2990		3660		
2-NaO-1: 4-N.Q. in H_2O	2180	3010		3820		
Naphthazarin in 10% EtOH + N/10 NaOH	1610		3230		(4070)	
	1750					
Hydroxy droserone	2030			3480	c. (4160)	
					c. 4360	
Hydroxy droserone triacetate		2960		c. 3880	c. 4170	

Values indicate wave numbers/mm. Values in () represent inflexions. All compounds were examined in alcoholic solution unless otherwise stated.

1: 2- and 1: 4- Naphthaquinone Derivatives.

As the number of 1 : 2-naphthaquinones is limited, a satisfactory comparison with the 1 : 4-naphthaquinones is impossible. Consequently a distinction between the 1 : 2 and 1 : 4 quinones is only possible for some of the simpler substances. From the preceding data it is evident that the introduction of hydroxyl groups in positions adjacent to the quinone oxygens considerably modifies the absorption curve, so that it is difficult to say whether the substance is a 1 : 2 or a 1 : 4 quinone. This is apparent in isonaphthazarin, whose absorption closely resembles that of the 1 : 2 quinone, though to infer a 1 : 2 structure would be contrary to Fieser's views.

At the same time it should be pointed out that since acetylation of the hydroxyl groups fixes the structure of the molecule by prevention of re-arrangements due to tautomeric hydrogen atoms, a distinction should be possible by an examination of the acetates. This is borne out by the acetates of the 1 : 4-naphthaquinones, which show absorption curves almost identical with that for 1 : 4 naphthaquinone. Unfortunately no acetates of 1 : 2-quinones were procurable, so that this point remains unconfirmed. Our conclusion is that, although the possibility of distinguishing between 1 : 2- and 1 : 4-naphthaquinones containing hydroxyl groups is doubtful, it may confidently be predicted that such a distinction will be possible by means of the acetates.



Ordinate: Log thickness (in mm.) of M/100,000 solution.
Abscissa: Wave numbers per mm.

Fig. 7.

— — — Hydroxy Droserone.
· · · · · · Hydroxy Droserone Triacetate.

From this it follows that, since the absorption curve for the triacetate of hydroxy droserone is similar to those for the acetates of hydroxy-1 : 4-naphthaquinones, it is almost certainly a derivative of a para quinone.

SUMMARY.

The qualitative absorption of a number of derivatives of 1 : 2- and 1 : 4-naphthaquinones has been examined.

The possibility of distinguishing by spectroscopic methods between 1 : 2 and 1 : 4 naphthaquinones, except in the case of those containing hydroxyl groups, has been established.

The structure of the triacetate of hydroxy droserone has been shown to be of the 1 : 4 quinone type.

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